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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have detected alterations of AKT2 and PI3K at kinase and protein levels in 32 of 80 human primary breast carcinomas. The majority of the cases with the kinase activation are ERα positive and late stage and high grade tumors. We have also shown that ERα interacts with p85 regulatory subunit of PI3K and activates PI3K/AKT2 pathway. Moreover, activated PI3K and AKT2 induce ERα transcriptional activity by phosphorylation of Serine-167 of ERα, suggesting that regulation between PI3K/AKT2 and ERα plays a pivotal role in mammary oncogenesis and that activation of PI3K/AKT2 contributes to ligand-independent breast cancer cell growth. In addition, we have demonstrated that AKT2 is activated by cellular stress and TNFα in human epithelial cells The activated AKT2 inhibits both JNK and p38 stress kinase activation and protects cells from UV, Heat shock and TNFα-induced apoptosis. AKT2-inhibited JNK1 activation is mediated by AKT2-induced NFκB.

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Introduction

The purpose of this project is to: 1) determine the incidence and clinicopathological significance of *PI3K/AKT2* alterations in breast cancer, 2) examine the role of overexpression of active and wild type PI3K/AKT2 in mammary cell transformation and 3) determine the role of PI3K/AKT2 in chemoresistance and as targets for breast cancer intervention.

Body:

During the last budget year, we have mainly focused on determination of incidence and clinicopathological significance of *PI3K/AKT2* alterations in breast cancer proposed in specific aim 1 and role of overexpression of active and wild type PI3K/AKT2 in mammary cell transformation proposed in specific aim 2.

1. PI3K and AKT2 are frequently altered in late stage and high grade human primary breast cancer

We have previously shown that the PI3K/AKT2 pathway is essential for cell survival and important in malignant transformation. Here, we demonstrate elevated kinase levels of AKT2 and PI3K in 32 of 80 primary breast carcinomas. Among 32 cases with PI3K and/or AKT2 activation, 23 are stages III and IV and 19 are poorly differentiated tumors. These data indicate that alterations of PI3K/AKT2 pathway associate with breast cancer progression rather than initiation. In addition, the majority of the cases with the activation are ERα positive (1). We are currently examining more cases in order to determine if PI3K and AKT2 could be a prognosis marker for breast cancer.

2. Feedback loop regulation between PI3K/AKT2 and ERα

The fact that breast cancer specimens with activation of PI3K/AKT2 are ERα positive prompted us to examine if AKT2 regulates ERα activity. We found that constitutively activated AKT2 or AKT2 activated by EGF- or IGF-1 promotes the transcriptional activity of ERα. This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ERα *in vitro* and *in vivo* , but not of a mutant ERα in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ERα induced by AKT2. However, AKT2-induced ERα activity was not inhibited by tamoxifen, but was completely abolished by ICI 164,384, implicating that AKT2-activated ERα contributes to tamoxifen-resistance. Moreover, we found that ERα binds to p85α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells, and subsequently activates PI3K/AKT2, suggesting ERα regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between ERα and PI3K/AKT2 pathway (ERα - PI3K/AKT2 - ERα) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth (1).

3. AKT2-induced cell survival is mediated by inhibition of JNK and p38 activation through inducing NFκB pathway

It has been well documented that JNK and p38 MAPKs are required for chemotherapeutic drug- and cellular stress-induced apoptosis (2-5). To determine if AKT2-induced cell survival is mediated by JNK/p38

pathway, we have examined if AKT2 is induced by stress and TNFα and whether activated AKT2 regulates JNK/p38. Our results show that AKT2 is significantly activated by UV-C irradiation, heat shock, and hyperosmolarity, as well as by TNFα through PI3K-dependent pathway. The activation of AKT2 inhibits UV- and TNFα–induced JNK and p38 activities. Moreover, AKT2 interacted with and phosphorylated IKKα. The phosphorylation of IKKα and activation of NFκB mediated AKT2 inhibition of JNK but not p38. Furthermore, PI3K inhibitor or dominant negative AKT2 significantly enhanced UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibited programmed cell death in response to UV and TNFα stimulation with accompanying decreased JNK and p38 activity (6). These results indicate that activated AKT2 protects cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence of Akt inhibits stress kinase JNK through activation of NFκB pathway.

4. Create mouse mammary tumor virus (MMTV)-AKT2 transgenic mouse

In collaboration with Dr. Barbara Vanderhyden at Ottawa Cancer Center, we have created 3 lines of mouse mammary tumor virus (MMTV) promoter driven-AKT2 transgenic mice, which have been observed for 1 months. No breast tumors have developed so far. We plan to maintain the mice for 1~1.5 years. We will also cross MMTV-AKT2 mice with MMTV-c-erbB2 mice, which will be obtained from Dr. William Muller at Ontario Cancer Institute.

Key Research Accomplishment

1 PI3K and AKT2 are frequently activated in late stage and high grade breast cancer.
2 ERα interacts with p85 regulatory subunit of PI3K and activates PI3K/AKT2 pathway.
3 PI3K/AKT2 activates ERα transactivation activity by phosphorylation of Ser-167 of ERα.
4 AKT2 is activated by stress and TNFα in epithelial cells.
5 Activated AKT2 inhibits stress-induced JNK and p38 activity by activation NFκB pathway.

Reportable Outcomes

1. Phosphoinositide-OH kinase/AKT2, activated in breast cancer, regulates and is induced by Estrogen Receptor α (ERα) via interaction between ERα and PI3K. *Cancer Res.* 61, 5985-5991, 2001
2. Inhibition of JNK by cellular stress- and tumor necrosis factor α-induced AKT2 through activation of the NFκB pathway in human epithelial Cells. J Biol Chem. 277(33):29973-2982, 2002.
3. Positive feedback regulation between Akt2 and MyoD during muscle differentiation. Cloning of Akt2 promoter. J Biol Chem. 277:23230-23235, 2002.

Conclusion

1. PI3K and AKT2 activation plays a pivotal role in breast cancer progression and tomaxifen resistance.
2. Feedback regulation between ERα and PI3K/AKT2 pathway is important for ligand-independent breast cancer growth.
3. Activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases JNK and p38 through activation of NFκB pathway. This work provided evidence to target AKT2 for overcome drug resistance in breast cancer which proposed in the Aim III.

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Appendices

1 Sun M, Paciga JE, Feldman RI, Yuan ZQ, Coppola D, Lu YY, Shelley SA, Nicosia SV, Cheng JQ. Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERα) via interaction between ERα and PI3K. *Cancer Res.*, 61:5985-5991, 2001.

2 Yuan ZQ, Feldman RI, Sun M, Olashaw NE, Coppola D, Sussman GE, Shelley SA, Nicosia SV, Cheng JQ. Inhibition of JNK by cellular stress- and TNFa-induced AKT2 through activation of the NFκB pathway in human epithelial cells. *J Biol Chem.* 277:29973-29982, 2002.

3. Kaneko S, Feldman RI, Yu L, Wu Z, Gritsko T, Shelley SA, Nicosia SV, Nobori T, Cheng JQ. Positive feedback regulation between Akt2 and MyoD during muscle differentiation. Cloning of Akt2 promoter. *J Biol Chem.* 277:23230-23235, 2002.

[CANCER RESEARCH 61, 5985–5991, August 15, 2001]

Advances in Brief

Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, Activated in Breast Cancer, Regulates and Is Induced by Estrogen Receptor α (ERα) via Interaction between ERα and PI3K[1]

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Abstract

We have shown previously that the AKT2 pathway is essential for cell survival and important in malignant transformation. In this study, we demonstrate elevated kinase levels of AKT2 and phosphatidylinositol-3-OH kinase (PI3K) in 32 of 80 primary breast carcinomas. The majority of the cases with the activation are estrogen receptor α (ERα) positive, which prompted us to examine whether AKT2 regulates ERα activity. We found that constitutively activated AKT2 or AKT2 activated by epidermal growth factor or insulin-like growth factor-1 promotes the transcriptional activity of ERα. This effect occurred in the absence or presence of estrogen. Activated AKT2 phosphorylates ERα *in vitro* and *in vivo*, but it does not phosphorylate a mutant ERα in which ser-167 was replaced by Ala. The PI3K inhibitor, wortmannin, abolishes both the phosphorylation and transcriptional activity of ERα induced by AKT2. However, AKT2-induced ERα activity was not inhibited by tamoxifen but was completely abolished by ICI 164,384, implicating that AKT2-activated ERα contributes to tamoxifen resistance. Moreover, we found that ERα binds to the p85α regulatory subunit of PI3K in the absence or presence of estradiol in epithelial cells and subsequently activates PI3K/AKT2, suggesting ERα regulation of PI3K/AKT2 through a nontranscriptional and ligand-independent mechanism. These data indicate that regulation between the ERα and PI3K/AKT2 pathway (ERα-PI3K/AKT2-ERα) may play an important role in pathogenesis of human breast cancer and could contribute to ligand-independent breast cancer cell growth.

Introduction

Breast cancer development and tumor growth are strongly associated with estrogens. The binding of an estrogen molecule to the ERα[3] induces a cascade of events, including the release of accessory proteins (*e.g.*, the heat-shock proteins), increased nuclear retention, DNA binding, and the transcription of estrogen-responsive genes, such as cyclin D1, c-myc, cathepsin D, and transforming growth factor-α that are known to stimulate mammary cell proliferation (1). ERα is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones. In common with other steroid hormone receptors, the ERα has a NH_2-terminal domain with a hormone-independent transcriptional activation function (AF-1), a central DNA-binding domain, and a COOH-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2; Refs. 2, 3). In addition to its ligand, estradiol, the ERα is also activated by several nonsteroidal growth factors including EGF and IGF1 through their cell membrane receptors and cytoplasmic signaling pathways such as MAPK signal transduction pathway (3, 4). Because of the role of ERα in promoting the growth and progression of breast cancers, considerable efforts are devoted to development of reagents to functionally inactivate ERα, so as to inhibit ERα-mediated gene expression and cell proliferation. Antiestrogens such as tamoxifen and ICI 164,384 antagonize the effects of estrogens by competing with estrogen for binding to ERα. Tamoxifen or its derivative 4-hydroxytamoxifen inhibits transcriptional activation by AF-2 but not AF-1 (5). ICI 164,384, on other hand, is a complete antagonist that blocks transcriptional activation by both AF-1 and AF-2 (6). However, approximately one-third of ERα-positive breast cancers fail to respond to antiestrogen treatment, which is thought to result from growth factor-induced ERα activity through activation of protein kinases resulting in phosphorylation of ERα (7).

It has been well documented that phosphorylation of ERα is essential for the activation of ERα after stimulation with its ligand and nonsteroidal growth factors (EGF and IGF1). The phosphorylation of ERα is observed at both serine and tyrosine residues. The serine residues are the predominant modified amino acids present in ERα, and four of these (Ser-104, Ser-106, Ser-118, and Ser-167) are clustered in the NH_2 terminus within the AF-1 region. Phosphorylation of ERα at Ser-118 is mediated by the Ras/MAPK pathway; therefore, activation of the MAPK pathway enables ligand-independent transactivation of ERα (4). There is evidence showing that Ser-167 is phosphorylated by several protein kinases, including casein kinase II and $pp90^{rsk1}$, which is important for DNA binding and transcriptional activation (8, 9). Phosphorylation of ERα on tyrosine 537, which is required for ERα dimerization and transactivation, by Src family tyrosine kinases *in vitro* has also been demonstrated. Moreover, protein kinase A has been shown to phosphorylate ERα at Ser-236 and regulate its dimerization (10).

In addition, recent studies (11) demonstrated that plasma membrane ERα plays a crucial role in transducing cellular signals. It has been convincingly shown that ERα activates G-protein-coupled receptor leading to the modulation of downstream pathways that have discrete cellular actions including membrane K^+ and Ca^{2+} channel activation and induction of protein kinase C and protein kinase A kinase activity (11). A recent study (12) demonstrated that estrogen activates p38 MAPK, resulting in the activation MAPK-protein kinase-2 and subsequent phosphorylation of heat shock protein 27. ERα has been also shown to interact with IGF1R and induce IGF1R and extracellular signal-regulated kinase activation (13).

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[3] The abbreviations used are: ERα, estrogen receptor α; PI3K, phosphatidylinositol-3-OH kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor; IGF1, insulin-like growth factor-1; GST, glutathione *S*-transferase; HEK, human embryonic kidney.

Akt, also called protein kinase B, has been identified as a direct target of PI3K (14). All of the three members, Akt/AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ, of this family are activated by growth factors in a PI3K-dependent manner (14–16). Numerous studies (17) showed that the Akt pathway is critical for cell survival by phosphorylation of a number of downstream proteins including BAD, caspase-9, Forkhead transcription factors, IKKα, Raf, and p21-activated protein kinase. Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies such as ovarian and pancreatic cancers (18–20). In this study, we demonstrate frequent activation of AKT2 and PI3K in human breast cancer. AKT2 phosphorylates ERα at Ser-167 and activates ERα-mediated transcription in a PI3K-dependent manner. ERα binds to the p85α subunit of PI3K in epithelial cells and activates the PI3K/AKT2 pathway in an estrogen-independent manner.

Materials and Methods

Tumor Specimens, Cell Lines, and Transfection. All of the 80 primary human breast cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained at least 70% tumor cells, as was confirmed by microscopic examination. The tissues were snap-frozen and stored at −70°C. ERα-negative epithelial HEK293 and COS7 cells and ERα-positive MCF7 and BG-1 cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS. The cells were seeded in 60-mm Petri dishes at a density of 8×10^5 cells/dish. Transfections were performed by calcium phosphate DNA precipitation or Lipofectamine Plus (Life Technologies, Inc.).

Immunoprecipitation and Western Blotting Analysis. The cells and frozen tumor tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (volume for volume) glycerol, 1% NP40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C before immunoprecipitation or Western blotting. The protein concentration in each tissue lysate was measured, and an equal amount of protein was analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-AKT2 (Upstate Biotechnology) antibody in the presence of 30 μl of protein A-protein G (2:1) agarose beads for 2 h at 4°C. The beads were washed once with 50 mM Tris-HCl (pH 7.5)-0.5 M LiCl-0.5% Triton X-10, twice with PBS, and once with 10 mM Tris-HCl (pH 7.5)-10 mM $MgCl_2$-10 mM $MnCl_2$-1 mM DTT, all containing 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. Immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein phosphorylation and expression were determined by probing Western blots of immunoprecipitates with anti-phospho-Akt-Ser473 (Cell Signaling) or anti-AKT2 antibody. Detection of antigen-bound antibody was carried out with the enhanced chemiluminescence Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** Akt kinase assay was performed as described previously (15). Briefly, the reaction was carried out in the presence of 10 μCi of [γ-^{32}P]ATP (NEN) and 3 μM cold ATP in 30 μl of buffer containing 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM DTT using histone H2B as substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein-loading buffer, and the mixture was separated in SDS-PAGE gels. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a Phosphorimager (Molecular Dynamics).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after dewaxing and rehydration. The Vectastain ABC Kit for sheep IgG (Vector Laboratories) was used to immunostain the tissue sections with phospho-S473 Akt antibody (catalogue number 06-801-MN; Upstate Biotechnology). Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to phospho-S473 Akt. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune sheep IgG on negative control sections.

PI3K Assay. PI3K was immunoprecipitated from the tumor tissue lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ-^{32}P]ATP, and 10 μg of L-a-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2; BIOMOL) for 20 min at 25°C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/methanol. Phosphorylated products were separated by TLC as described previously (21). The conversion of PI-4,5-P_2 to PI-3,4,5-P_3 was determined by autoradiography and quantitated by using a Phosphorimager. Average readings of the kinase activity 3-fold higher than that in normal ovarian tissue was considered as elevated PI3K activity.

Expression Constructs and GST Fusion Protein. HA epitope-tagged constitutively active, wild-type, and dominant-negative AKT2 were prepared as described previously (21). The p110α and p85α of PI3K expression constructs were gifts from Dr. Julian Downward (Imperial Cancer Research Fund, London, United Kingdom). The mammalian expression construct of ERα-S167A was kindly provided by Dr. Benita S. Katzenellenbogen (University of Illinois, Urbana, IL). The GST-ERα and GST-ERα-S167A were created by PCR and inserted into pcDNA3 and pEGX-4T (Pharmacia) vectors, respectively. GST-ERα fusion proteins were purified as described previously (21).

***In Vivo* [^{32}P]P_i Cell Labeling.** Transfected COS7 and nontransfected MCF7 cells were labeled with [^{32}P]P_i (0.5 mCi/ml) in MEM without phosphate, serum, and phenol red for 4 h and lysed. ERα was immunoprecipitated with monoclonal anti-ERα or anti-myc antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membranes. Phosphorylated ERα was detected by autoradiography and quantitated by using Molecular Dynamics Phosphorimager with ImageQuant software.

Reporter Assay. HEK293 and MCF7 cells (8×10^5) were seeded in a 60-mm plate. The cells were cotransfected with the luciferase reporter plasmid (2ERE-MpG12), wild-type, constitutively active, or dominant-negative AKT2 and ERα, as well as pCMV-βgal plasmid as an internal control. The amount of DNA in each transfection was kept constant by the addition of empty pcDNA3 vector. Luciferase and β-galactosidase activities were determined 48 h after transfection according to the manufacturer's procedure (Promega). Luciferase activity was corrected for transfection efficiency by using the control β-galactosidase activity. All of the experiments were performed in triplicate from independent cell cultures.

Results and Discussion

Frequent Activation of AKT2 in Breast Carcinoma. We have demonstrated previously (15, 20) that AKT2, like AKT1, is activated by a number of mitogenic growth factors in a PI3K-dependent manner and that AKT2 kinase activity is frequently elevated in human ovarian tumors. To examine whether AKT2 is activated in human primary breast cancer, we performed *in vitro* kinase assays in 80 human breast carcinoma specimens, including 58 ductal infiltrating adenocarcinomas, 16 lobular carcinomas, and six mixed tumors. Lysates from tumor specimens were incubated with anti-AKT2 antibody, which specifically reacts with AKT2 (20). The immunoprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. The results revealed an elevated level of AKT2 kinase in 32 of specimens (40%), including 29 cases with ductal infiltrating carcinoma, two lobular, and one mixed tumor (Fig. 1*A*). To further demonstrate AKT2 activation in breast cancer, we performed Western blot analyses of tumor lysates with phospho-Ser-473 antibody, a phosphorylation site that is critical for activation of three isoforms of Akt (17). To avoid the cross-reaction, the tumor lysates were incubated with anti-AKT2 antibody. The AKT2 immunoprecipitates were separated by SDS-PAGE and probed with phospho-Ser473 antibody. Phosphorylated AKT2 was detected only in breast tumors with elevated AKT2 kinase

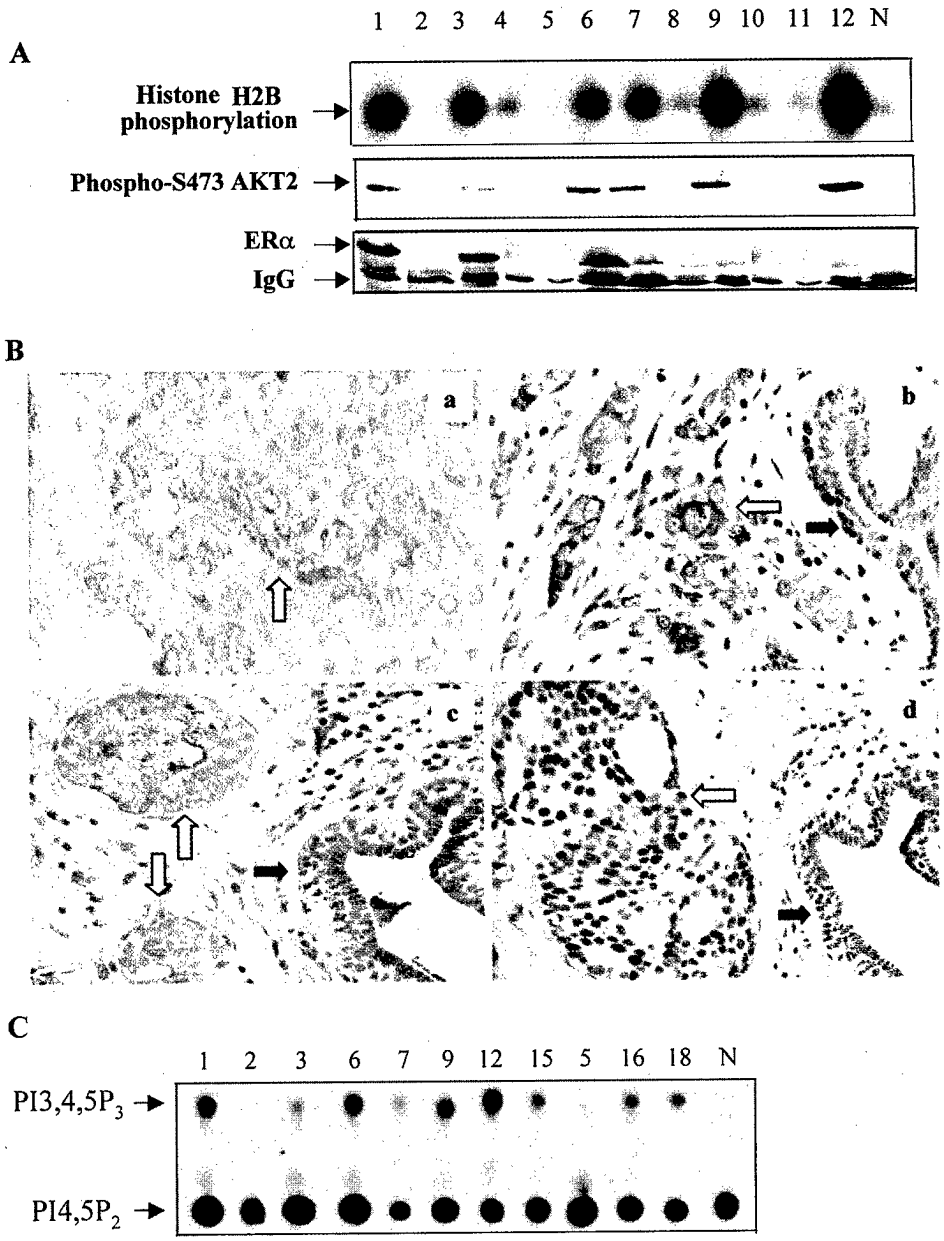

Fig. 1. Activation of AKT2 in human primary breast cancers. *A* (*top panel*), *in vitro* kinase assays of immunoprecipitated AKT2 from representative frozen breast tumor specimens. Normal mammary tissue (*N*) was used as a control. *Bottom panels*, Western blot analyses of AKT2 and ERα immunoprecipitates with anti-phospho-Ser473 Akt and anti-ERα antibodies, respectively. *B*, immunochemical staining of the paraffin sections prepared from primary breast adenocarcinomas with anti-phospho-S473 Akt (*a–c*) and anti-ERα (*d*) antibodies. Strong staining with both antibodies was observed in tumor cells (*white arrows*), whereas weak immunoreaction was detected in stromal tissue and adjacent ductal epithelium (*black arrows*). Photomicrographs *c* and *d* are the same specimen but different sections. *C*, *In vitro* PI3K assay of anti-p85 immunoprecipitates from 11 tumor and one normal specimen. The specimen numbers correspond to the same tumors shown in *A*.

activity (Fig. 1*A*). Because stromal tissues account for approximately 20–30% of the tumor specimens used in this study, we examined whether the activation of AKT2 is derived from the tumor cells or the stromal tissues by immunostaining paraffin sections with a phospho-Ser473 Akt antibody. Positive staining of tumor cells was detected in all of the 32 cases with AKT2 activation, whereas no staining was observed in normal ductal epithelial cells (Fig. 1*B*). These data suggest that activation of AKT2 is a common occurrence in human breast cancer.

Because AKT2 is a downstream target of PI3K, which is activated in colon and ovarian carcinoma (20, 22, 23), we next examined the PI3K activity in breast tumors by *in vitro* PI3K assay. Because of the fact that all of the tumors with elevated PI3K activity result in activation of Akt (20, 22–24), immunoprecipitation with a pan-p85 antibody was performed in 58 breast tumor specimens, including 32 with AKT2 activation and, as control, 26 without AKT2 activation. The ability to convert PI-4,5-P_2 to PI-3,4,5-P_3 was determined. Elevated PI3K activity was detected in all of the 32 specimens that exhibited AKT2 activation. No PI3K activation was observed in 26 specimens without AKT2 activation (Fig. 1*C*), indicating that activation of AKT2 in breast cancer predominantly results from PI3K activation. Moreover, Western blot and immunohistochemistry analyses with anti-ERα antibody revealed that 88% of the cases (28 of 32) with PI3K/AKT2 activation showed strong ERα positive (Fig. 1, *A* and *B*), whereas only 54% of the cases (14 of 26) without PI3K/AKT2 activation exhibited positive ERα, suggesting that activated PI3K/AKT2 could be involved in the regulation of ERα activity in breast cancer cells. In addition, the majority of cases with AKT2 activation are late stage (23 of 32 at stages III and IV) and poorly differentiated tumors (19 of 32), indicating that PI3K/AKT2 activation in breast cancer may be associated with tumor progression rather than initiation.

AKT2 Activates ERα-mediated Transcription in a Ligand-independent Manner. Previous studies (1, 25) have shown that MAPK is activated in breast cancer and contributes to estrogen-independent breast tumor cell growth by direct phosphorylation of ERα. Moreover, several other signal molecules, including protein kinase A, casein kinase II, $pp90^{rsk1}$, and MEKK1/p38, have been

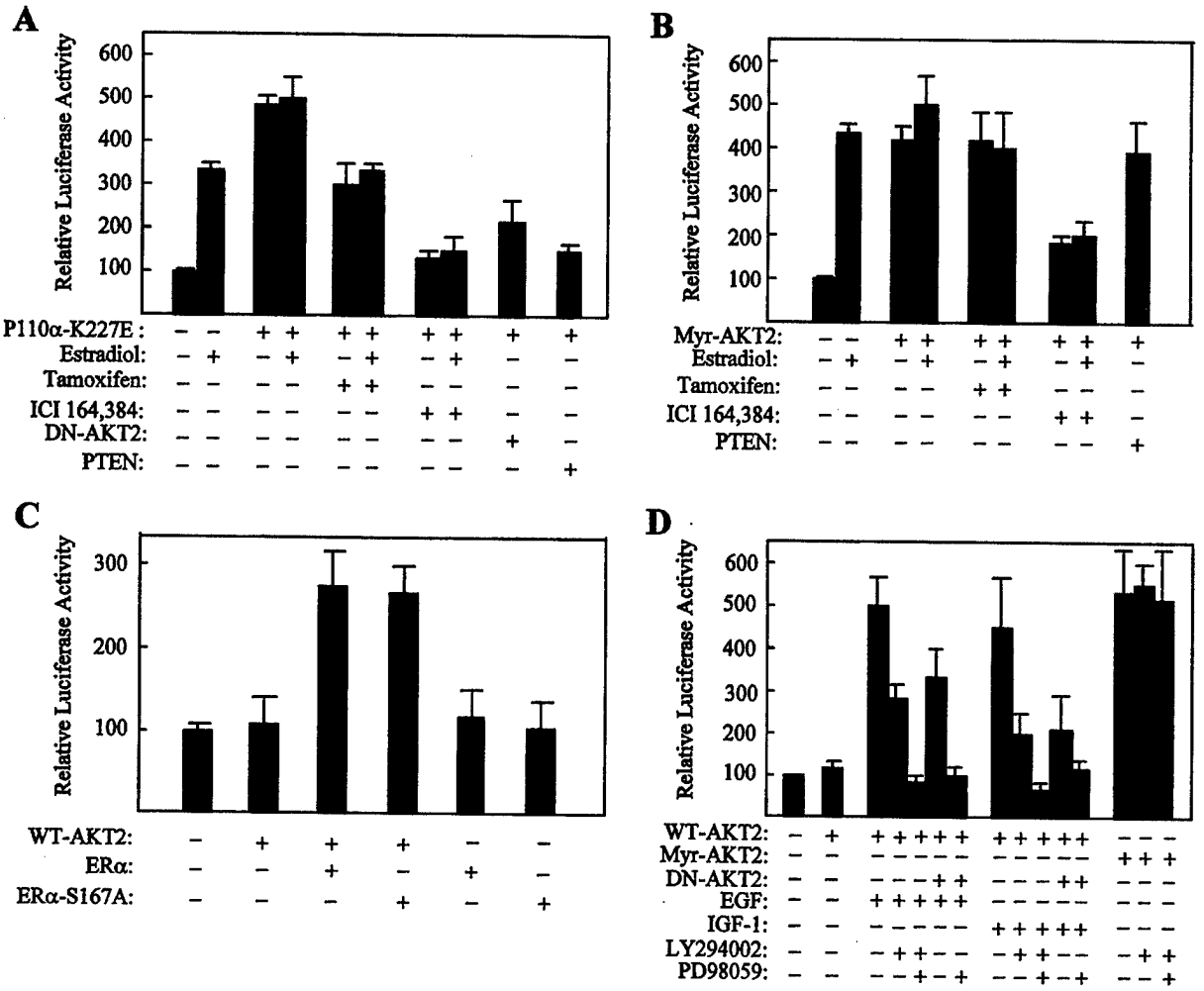

Fig. 2. AKT2 and PI3K activate ERα transcriptional activity. *A–D*, reporter assays: MCF-7 cells were transfected with ERE2-TK-LUC reporter, β-galactosidase, and indicated expression constructs. After 36 h of transfection, the cells were serum-starved overnight and treated with indicated agents. Luciferase activity was normalized to β-galactosidase activity.

shown to activate ERα-mediated transcription, possibly resulting in hormone-independent tumor cell growth (1, 8–10, 26). Because AKT2 and PI3K are frequently activated in breast cancer and the majority of cases with AKT2 activation are ERα positive, we investigated whether AKT2 and PI3K regulate ERα-mediated transcription. ERα-positive MCF7 breast cancer cells were transiently transfected with a reporter construct containing a luciferase gene regulated by two estrogen response elements (ERE2-TK-LUC) and a plasmid expressing β-galactosidase that allows the luciferase data to be normalized for transfection efficiency. In addition, the cells were transfected with expression constructs for constitutively activated p110α (p110α-K227E) subunit of PI3K, wild-type, constitutively activated, and dominant-negative AKT2 or vector alone. As shown in Fig. 2, p110α-K227E or myr-AKT2 increased ERE2-TK-LUC activity 3–4-fold in the absence of estradiol. Constitutively activated p110α-induced reporter activity was attenuated by dominant-negative mutant AKT2 (Fig. 2*A*). Tamoxifen (4-hydroxytamoxifen), an antiestrogen reagent that inhibits transcriptional activation by AF2 but not through AF1 (5), abolished estradiol-enhanced transcription but had no effects on p110α-K227E and myr-AKT2-stimulated ERα activity (Fig. 2, *A* and *B*), suggesting that PI3K/AKT2-increased ERα transcriptional activity is regulated by phosphorylation of ERα within the AF1 region and could be involved in tamoxifen resistance.

ICI 164,384, which causes rapid degradation of ERα (6, 27), completely blocked PI3K- and AKT2-induced reporter activity. PTEN, a tumor suppressor encoding a lipid phosphatase that negatively regulates PI3K, inhibited constitutively active p110-induced ERα-mediated transcription but had no effect on constitutively activated AKT2-stimulated ERα activity (Fig. 2*B*).

Moreover, we have observed that exogenous expression of ERα in ERα-positive MCF7 cells increased wild-type AKT2-induced ERE2-TK-LUC activity 2–3-fold as compared with cells transfected with wild-type AKT2 alone (Fig. 2*C*), implying that ERα might activate AKT2 kinase and subsequently enhance its own transcriptional activity (see below). Taken collectively, these data indicate that PI3K/AKT2-activated ERα-mediated transcription is estrogen-independent and that the frequently elevated level of PI3K/AKT2 kinase in primary breast cancer could relate the refractoriness of hormone therapy.

AKT2 Mediates Growth Factor-induced ERα Transcriptional Activity. A very recent study (28) showed that Akt1 mediates the estrogenic functions of EGF and IGF1. Next, we examined the possible role of AKT2 in growth factor-induced ERα activation. ERα-positive MCF7 cells were transfected with ERE2-TK-LUC and dominant-negative, wild-type, or constitutively activated AKT2 or vector alone and were treated with or without either 100 ng/ml EGF or 50 ng/ml IGF1 (Fig. 2*D*). Treatment with the growth factors resulted in an approximately 4.5-fold increase in ERα-mediated transcriptional activity. The EGF- and IGF1-induced reporter activity was partially abrogated by dominant-negative AKT2 or PI3K inhibitor LY294002 and completely blocked by the combination of PI3K and MAPK inhibitors (LY294002 and PD98059). However, the combined inhibitors had no effect on constitutively activated AKT2-induced reporter

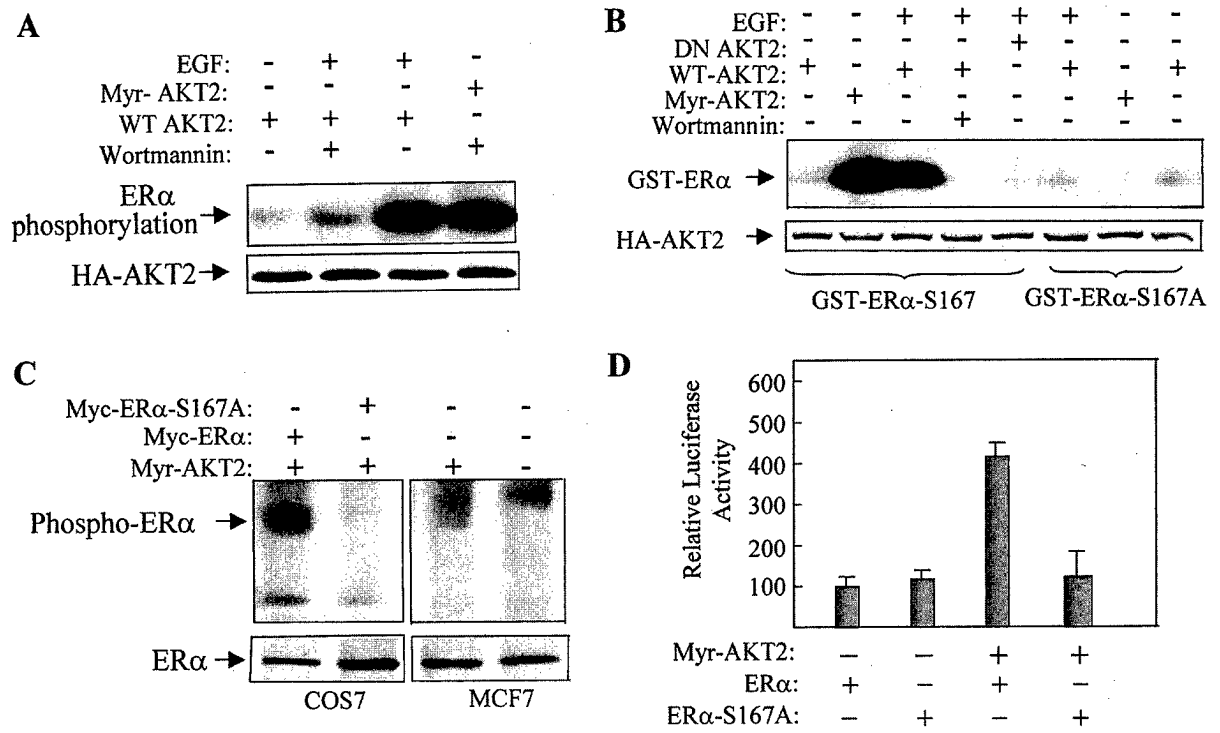

Fig. 3. AKT2 phosphorylates ERα on serine-167 *in vitro* and *in vivo*. *In vitro* AKT2 kinase assay of the immunoprecipitates from HEK293 cells transfected with indicated expression constructs. Full length of human recombinant ERα (*A*), GST-ERα-S167, and GST-ERα-S167A (*B*) were used as substrates. *C*, COS7 and MCF7 cells were transfected with indicated plasmids and incubated with $[^{32}P]P_i$ for 4 h. Immunoprecipitates were prepared with anti-myc (*left*) or anti-ERα (*right*) antibody and separated by SDS-PAGE. After transfer, the membrane was exposed to a film (*top*) and detected with anti-ERα antibody (*bottom*). *D*, AKT2 phosphorylation of serine-167 is essential for AKT2-induced ERα transcriptional activity. Luciferase reporter assay of HEK293 cells transfected with ERE2-TK-LUC, wild-type ERα, or ERα-S167A, β-galactosidase, and myr-AKT2.

activity. These results suggest that the "steroid-independent activation" of ERα by growth factors is mediated by the PI3K/AKT2 pathway, in addition to MAPK, PKA, casein kinase II, and $pp90^{rsk1}$.

AKT2 Phosphorylated Serine-167 of ERα *in Vitro* and *in Vivo*. Phosphorylation of ERα has been shown to be an important mechanism by which ERα activity is regulated. ERα is hyperphosphorylated on multiple sites in response to hormone binding and growth factor stimulation (1–4). Transcriptional activation by growth factors has been shown to require AF-1 but not AF-2 (1, 8–10). There is evidence to suggest that EGF and IGF-1 induce MAPK and $pp90^{rsk1}$/casine kinase II activity leading to phosphorylation of serine-118 and serine-167, respectively, in AF-1 region (4, 5, 8–10). To examine whether AKT2 phosphorylates ERα *in vitro*, HEK293 cells were transfected with HA-tagged wild-type and constitutively activated AKT2, and immunoprecipitation was prepared with anti-HA antibody. *In vitro* AKT2 kinase assays, using full length of human recombinant ERα as substrate, revealed that constitutively activated AKT2 and EGF-induced AKT2 strongly phosphorylated hERα. The ERα phosphorylation that was induced by EGF-stimulated AKT2 was abrogated by wortmannin (Fig. 3*A*).

To determine whether AKT2 phosphorylates ERα *in vivo*, MCF7 cells were transfected with constitutively activated AKT2 or pcDNA3 vector alone and labeled with $[^{32}P]P_i$. The cell lysates were incubated with anti-ERα antibody, and the immunoprecipitates were separated on SDS-PAGE. ERα was highly phosphorylated in constitutively activated AKT2-transfected cells but in the cells transfected with vector alone (Fig. 3*C*). These data indicate that AKT2 phosphorylated ERα both *in vitro* and *in vivo*.

Martin *et al.* (28) recently demonstrated that EGF- and IGF1-induced Akt1 potentiates the AF-1 function of ERα, possibly through the phosphorylation of serine residues. There are four serine residues (Ser-104, Ser-106, Ser-118, and Ser-167) in the AF-1 region of the receptor that are predominantly phosphorylated in response to estrogen and growth factor stimulation (1–4). We examined the ERα protein sequence and found that serine-167 (^{162}**RERLAS**^{167}) is a putative AKT2 phosphorylation site. Constructs expressing GST-fused wild-type and mutant (S167A) AF-1 region were created. *In vitro* kinase assays revealed that myr-AKT2 and EGF-stimulated AKT2 strongly phosphorylated GST-ERα-S167 but not GST-ERα-S167A mutant (Fig. 3*B*). The EGF-induced AKT2 phosphorylation of ERα is blocked by wortmannin. To examine whether AKT2 phosphorylates serine-167 *in vivo*, COS7 cells were transfected with myc-tagged wild-type and mutant (S167A) human ERα expression constructs together with constitutively activated AKT2. After 36 h of transfection, the cells were incubated with $[^{32}P]P_i$ and immunoprecipitated with anti-myc antibody. As demonstrated in Fig. 3*C*, constitutively active AKT2 phosphorylated wild-type ERα but not the ERα-S167A mutant *in vivo*, suggesting that serine-167 of ERα is a physiological substrate for AKT2.

Previous studies (29) showed that serine-167 is important for ERα transcriptional activity. To further examine whether AKT2-activated ERα transcriptional activity depends upon phosphorylation of serine-167, reporter assays were carried out in HEK293 cells transfected with ERE2-TK-LUC, constitutively activated AKT2, and ERα-S167A or wild-type ERα. Fig. 3*D* shows that ERα-S167A had no ability to mediate constitutively activated AKT2-induced ERE2-TK-LUC reporter activity, indicating that AKT2 regulates ERα-mediated transcription through phosphorylation of serine-167.

ERα Binds To and Activates PI3K/AKT2 in Epithelial Cells via a Ligand-independent Mechanism. Recent studies (30, 31) demonstrated that ERα binds to the p85α regulatory subunit of PI3K after estradiol treatment, leading to the activation of PI3K/Akt and endo-

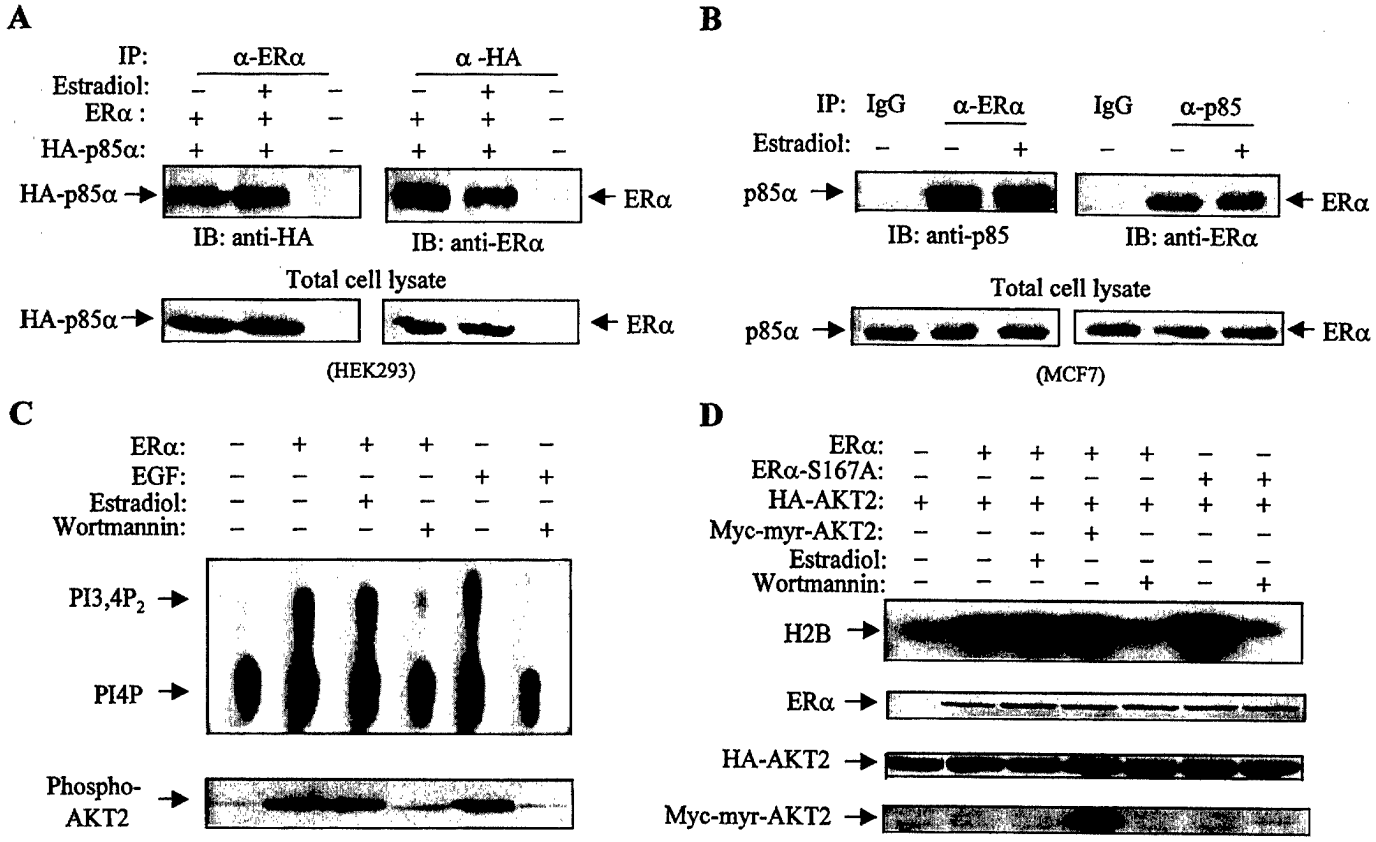

Fig. 4. ERα interacts with p85α and activates the PI3K/AKT2 pathway in human epithelial cells. Coimmunoprecipitation of ERα and p85α in (*A*) HEK293 cells cotransfected with HA-p85α/ERα and in (*B*) nontransfected MCF7 cells. *Top*, coimmunoprecipitation; *bottom*, Western blot of total cell lysates. *C*, *in vitro* PI3K assay (*top*) of HEK293 cells transfected and treated with indicated plasmid and agents. *Bottom*, Western blotting analysis of AKT2 immunoprecipitates with phospho-S473 Akt antibody. *D*, *in vitro* kinase assay (*top*) of HA-AKT2 immunoprecipitates prepared from HEK293 cells transfected with indicated expression constructs, using histone H2B as a substrate. *Panels 2–4*, Western blots of transfected HEK293 cell lysates detected with anti-ERα, anti-HA, or anti-myc antibody.

thelial nitric oxide synthase in endothelial cells. In the absence of estradiol, ERα failed to bind and activate PI3K, indicating that ERα-associated PI3K in endothelial cells is estrogen-dependent (30). Next, we examined whether ERα binds to and activates PI3K/AKT2 in epithelial cells. ERα/HA-p85α-transfected HEK293 and nontransfected ERα-positive MCF7 cells were immunoprecipitated with anti-ERα and detected with anti-HA or anti-p85α antibody or *vice versa*. As shown in Fig. 4*A* and *B*, ERα constitutively associated with p85α, and this interaction was unaffected by estradiol treatment. In addition, *in vitro* PI3K assays revealed that expression of ERα in HEK 293 cells significantly induced PI3K activity in the absence or presence of estradiol (Fig. 4*C*). These data suggest that ERα binding to and activating PI3K is ligand-independent in epithelial cells.

Next, we examined whether ERα activates AKT2 and whether this activation is dependent on AKT2 phosphorylation. ERα-negative HEK293 cells were transfected with ERα or ERα-S167A, together with HA-AKT2. *In vitro* AKT2 kinase assays revealed that ERα significantly activates AKT2 in the absence of estradiol. Additional estradiol treatment did not further enhance ERα-induced AKT2 activation. The PI3K inhibitor, wortmannin, completely abolished the activation. Interestingly, ERα-S167A activated AKT2 at a similar level to that of wild-type ERα. Coexpression of myc-tagged constitutively active AKT2 (Myc-myr-AKT2) and ERα had the same effect on wild-type AKT2 activation as that of expression of ERα alone (Fig. 4*D*). These results indicate that activation of AKT2 by ERα is through PI3K and independent of ERα phosphorylation by PI3K/AKT2.

In summary, we demonstrate in this study that AKT2 and PI3K are frequently activated in primary human breast carcinoma. The PI3K/AKT2 pathway regulates ERα transcriptional activity by phosphorylation of serine-167 *in vitro* and *in vivo*, and ERα activates PI3K/AKT2 kinase by binding to p85α in a ligand-independent manner in epithelial cells. This study suggests that the PI3K/AKT2 pathway may play a pivotal role in estrogen-independent breast cancer cell growth and tamoxifen-resistance; therefore, it could represent an important therapeutic target in human breast cancer.

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Inhibition of JNK by Cellular Stress- and Tumor Necrosis Factor α-induced AKT2 through Activation of the NFκB Pathway in Human Epithelial Cells*

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Previous studies have demonstrated that AKT1 and AKT3 are activated by heat shock and oxidative stress via both phosphatidylinositol 3-kinase-dependent and -independent pathways. However, the activation and role of AKT2 in the stress response have not been fully elucidated. In this study, we show that AKT2 in epithelial cells is activated by UV-C irradiation, heat shock, and hyperosmolarity as well as by tumor necrosis factor α (TNFα) through a phosphatidylinositol 3-kinase-dependent pathway. The activation of AKT2 inhibits UV- and TNFα-induced c-Jun N-terminal kinase (JNK) and p38 activities that have been shown to be required for stress- and TNFα-induced programmed cell death. Moreover, AKT2 interacts with and phosphorylates IκB kinase α. The phosphorylation of IκB kinase α and activation of NFκB mediates AKT2 inhibition of JNK but not p38. Furthermore, phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 significantly enhances UV- and TNFα-induced apoptosis, whereas expression of constitutively active AKT2 inhibits programmed cell death in response to UV and TNFα stimulation with an accompanying decreased JNK and p38 activity. These results indicate that activated AKT2 protects epithelial cells from stress- and TNFα-induced apoptosis by inhibition of stress kinases and provide the first evidence that AKT inhibits stress kinase JNK through activation of the NFκB pathway.

Exposure of cells to environmental stress results in the activation of several signal transduction pathways including the MEKK4/MKK7/JNK,[1] MKK3/MKK6/p38, and IκB kinase (IKK)/IκB/NFκB cascades. Stress-induced clustering and internalization of cell surface receptors, such as those for platelet-derived growth factor, tumor necrosis factor α (TNFα), epidermal growth factor, and insulin-like growth factor 1 (IGF1), mediate stress-kinase activation (1–3). Recent studies suggest that nearly all stress stimuli activate phosphatidylinositol 3-kinase (PI3K) (1), and of the downstream targets of PI3K, AKT is thought to play an essential role in the cellular response to stress.

AKT, also termed protein kinase B or RAC kinase, represents a family of PI3K-regulated serine/threonine kinases (4, 5). Three different isoforms of AKT have been identified, AKT1/protein kinase Bα (AKT1), AKT2/protein kinase Bβ (AKT2), and AKT3/protein kinase Bγ (AKT3), all of which are activated by growth factors in a PI3K-dependent manner (4–9). Full activation of the AKTs requires their phosphorylation at Thr^{308} (AKT1), Thr^{309} (AKT2), or Thr^{305} (AKT3) in the activation loop and Ser^{473} (AKT1), Ser^{474} (AKT2), or Ser^{472} (AKT3) in the C-terminal activation domain (9). AKT1, the most studied isoform, which was originally designated as AKT, suppresses apoptosis induced by a variety of stimuli, including growth factor withdrawal and loss of cell adhesion. Possible mechanisms by which AKT1 promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (10, 11). AKT1 also phosphorylates and inactivates the Forkhead transcription factors, an event that results in the reduced expression of the cell cycle inhibitor, $p27^{Kip1}$, and the Fas ligand (12–14). Via phosphorylation of IKK, AKT1 also activates NFκB, a transcription factor that has been implicated in cell survival (15, 16).

Two separate studies demonstrated that AKT1 is activated when NIH 3T3 fibroblasts are stressed in a variety of ways (17, 18). Based on data showing that PI3K inhibitors do not prevent AKT1 activation by stress, these studies concluded that stress-induced AKT1 activation was PI3K-independent. Other studies, however, found that PI3K activity was required for AKT1 activation by heat shock or oxidative stress in Swiss 3T3 cells (19, 20). It has been suggested that certain cellular stresses activate AKT1 and AKT3 but not AKT2 (19), a finding that is consistent with the different functions of the AKTs as revealed by studies of mice lacking AKT1 or AKT2 (21–23). Nevertheless, activation of AKT2 by stress and the role of AKT2 in the stress response have yet to be fully explored. The data presented here show that AKT2 is significantly activated by stress stimuli (*e.g.* UV irradiation, heat shock, and hyperosmolarity) and by TNFα in human epithelial cells but not in fibroblasts. Stress-induced AKT2 activation in epithelial cells is completely blocked by inhibitors of PI3K. When activated by stress, AKT2 inhibits JNK and p38 activities that are required for stress-induced apoptosis. In addition, AKT2 binds to and phosphoryl-

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[1] The abbreviations used are: JNK, c-Jun N-terminal kinase; TNFα, tumor necrosis factor α; IGF1, insulin-like growth factor 1; PI3K, phosphatidylinositol 3-kinase; HA, hemagglutinin; IKK, IκB kinase; NIK, NFκB-inducing kinase; GST, glutathione *S*-transferase; HEK, human embryonic kidney; TUNEL assay, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling assay.

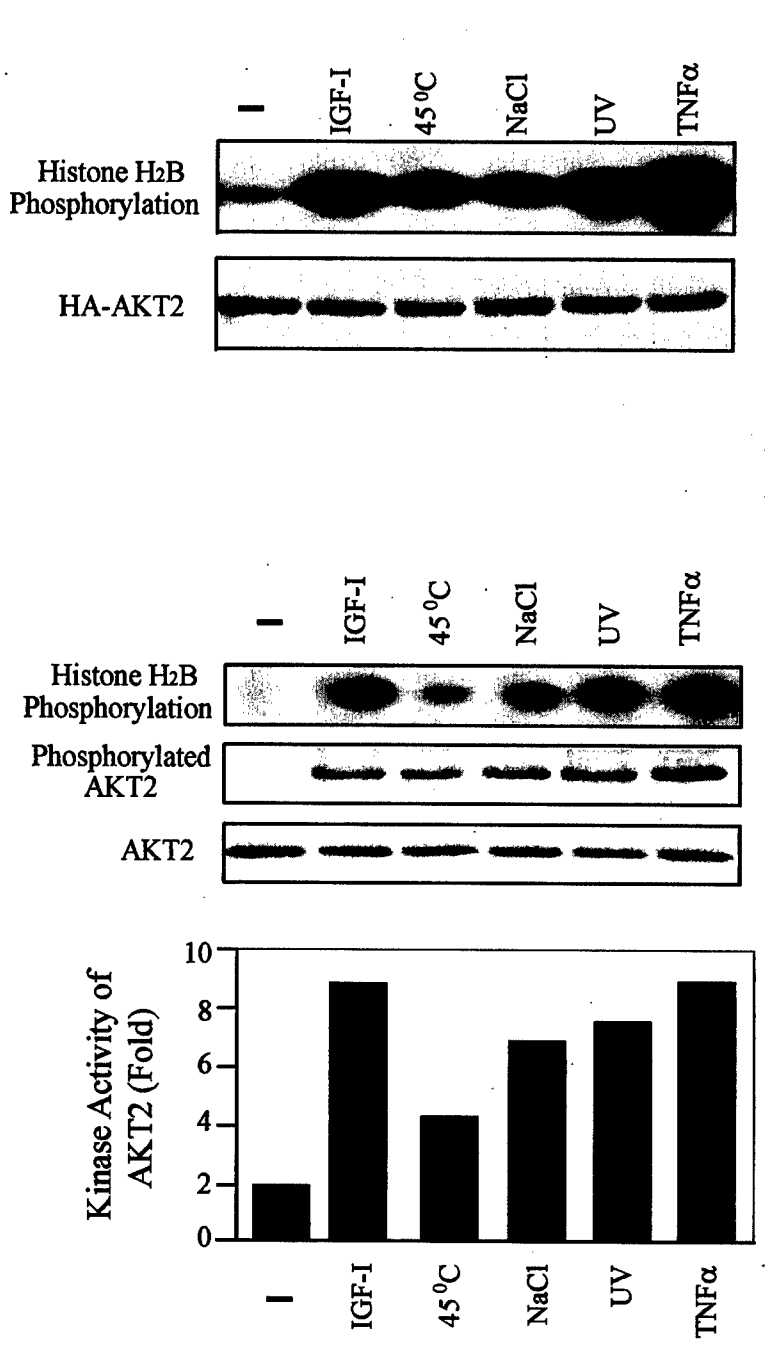


FIG. 1. **AKT2 is activated by cellular stress and TNFα.** *A*, *in vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transiently transfected with HA-AKT2. Cells were exposed to 100 ng/ml IGF-1 (15 min), heat shock (45 °C for 20 min), 0.4 M NaCl (15 min), 40 J/m^2 UV-C (254 nm), or TNFα 20 ng/ml (15 min), and AKT2 activity was determined by *in vitro* kinase assay using histone H2B as substrate. *B*, OVCAR3 cells were treated with the indicated stimuli and immunoprecipitated with anti-AKT2 antibody. The immunoprecipitates were subjected to *in vitro* kinase assay (*upper*) and Western blotting analyses with anti-phospho-Ser473 AKT (*middle*), or anti-AKT2 (*lower*) antibody. The *bottom panel* shows relative AKT2 kinase activity quantified by phosphorimaging. Each experiment was repeated three times.

ates IKKα and, consequently, activates NFκB, resulting in inhibition of programmed cell death in response to stress stimuli. Moreover, AKT2-induced NFκB activation is required for the inhibition of JNK, but not p38, activity.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfection, and Stimulation—The human epithelial cancer cell lines, A2780, OVCAR3, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells/dish. After incubation overnight, the cells were transfected with 2 μg of DNA/dish using LipofectAMINE Plus (Invitrogen). After 36 h of the transfection, the cells were serum-starved overnight and stimulated with UV-C irradiation, heat (45 °C), 0.4 M NaCl, or 20–50 ng/ml TNFα.

Expression Constructs—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2, constitutively active HA-Myr-AKT2, and dominant negative HA-E299K-AKT2 have been described (24). The HA-JNK1 construct was kindly provided by Michael Karin (School of Medicine, University of California at San Diego). GST-c-Jun-(1–79) and pCMV-FLAG-p38 were gifts from Roger J. Davis (School of Medicine, University of Massachusetts). The constructs used in the study of the NFκB pathway were prepared as previously described (25).

Immunoprecipitation and Immunoblotting—Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerol phosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4 °C before immunoprecipitation or Western blotting. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. After the removal of the beads by centrifugation, lysates were incubated with anti-HA monoclonal antibody 12CA5 (Roche Molecular Biochemicals), anti-FLAG antibody (Sigma), or anti-AKT2 antibody (Santa Cruz Biotechnology) in the presence of 30 μl of protein A-protein G (2:1)-agarose beads for 2 h at 4 °C. The beads were washed once with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10, twice with phosphate-buffered saline, and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10

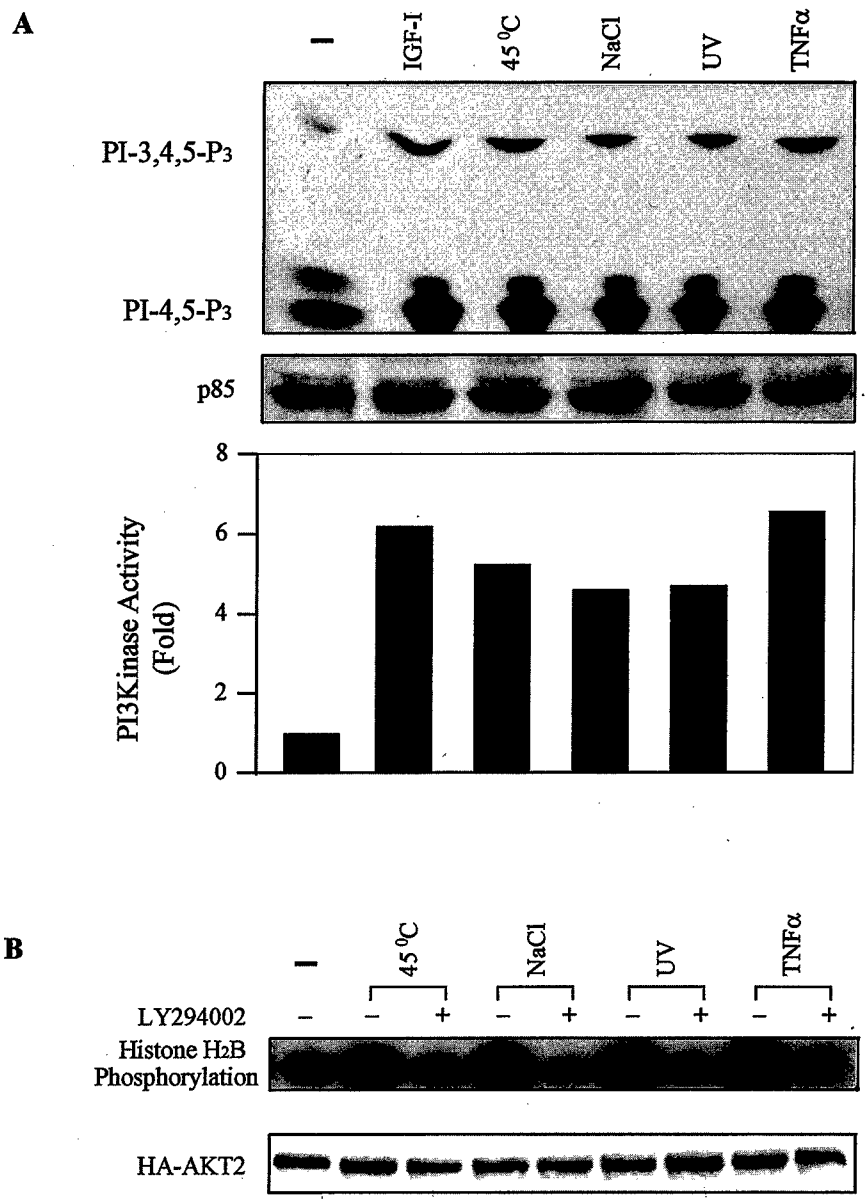


FIG. 2. **Activation of AKT2 by cellular stress and TNFα is PI3K-dependent.** *A*, *in vitro* PI3K assay. HA-AKT2-transfected HEK293 cells were exposed to the indicated stimuli. *Upper panel*, PI3K immunoprecipitates were prepared with anti-pan-p85 antibody and assayed for PI3K activity. The *middle panel* shows the p85 protein level using anti-p85 antibody, and the *bottom panel* represents the relative PI3K activity quantified by phosphorimaging. *B*, HA-AKT2-transfected A2780 cells were treated with LY294002 for 30 min before exposure to indicated stimuli. HA-AKT2 immunoprecipitates were subjected to *in vitro* kinase assay. Results were confirmed by four independent experiments. *PI-3,4,5-P_3*, phosphatidylinositol 3,4,5-trisphosphate; *PI-4,5-P_3*, phosphatidylinositol 4,5-trisphosphate.

mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerol phosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the antibodies described above or with the appropriate antibodies as noted in figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting Analysis System (Amersham Biosciences).

In Vitro Protein Kinase Assay—Protein kinase assays were performed as previously described (26, 27). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P] ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. Histone H2B was used as exogenous substrate. After incubation at room temperature for 30 min, the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

PI3K Assay—PI3K was immunoprecipitated from the cell lysates with pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold phosphate-buffered saline, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in the immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [γ-^{32}P]ATP and 10 μg L-α-phosphatidylinositol 4,5-bisphosphate (Biomol) for 20 min at 25 °C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/MeOH, and phosphorylated products were separated by thin-layer chromatography as previously described (24). The conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate was detected by autoradiography and quantitated with a PhosphorImager.

NFκB Transcriptional Activation Analysis—HEK293 cells were seeded in 60-mm dishes and transfected with 1.5 μg of NFκB reporter plasmid (pElam-luc), 0.8 μg of pSV2-β-gal, and different forms (wild type, constitutively active, or dominant-negative) of HA-AKT2 or vector alone. The total amount of DNA transfected was increased to 6 μg with empty vector DNA. After serum starvation overnight, the cells were treated with UV (40 J/m^2) or TNFα (20 ng/ml) and lysed with 400 μl/dish of reporter lysis buffer (Tropix). The cell lysates were cleared by centrifugation for 2 min at 4 °C. Luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL) Assay—AKT2 stably transfected A2780 cells were seeded into 60-mm dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum for 24 h and pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. Apoptosis was determined by TUNEL using an *in situ* cell death detection kit (Roche Molecular Biochemicals). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in phosphate-

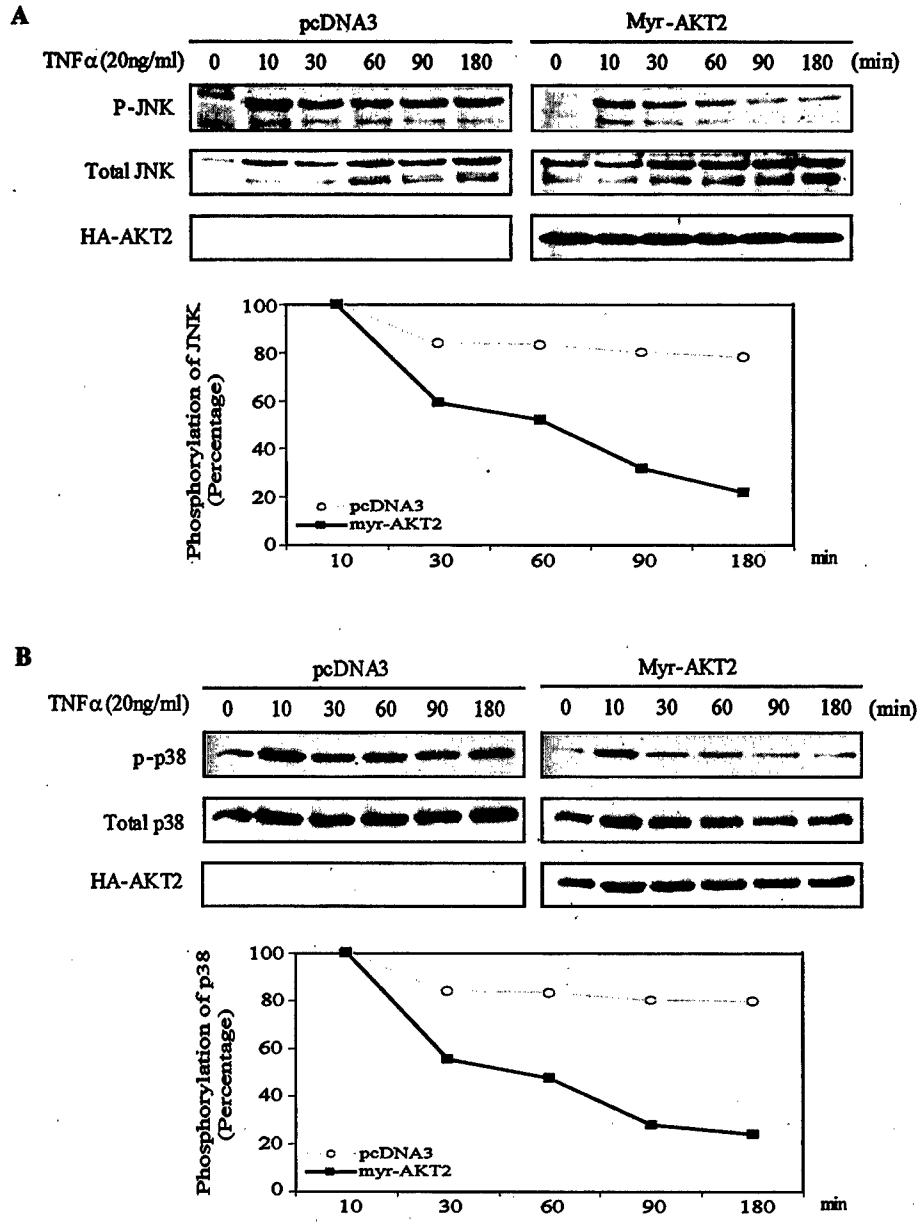


FIG. 3. **AKT2 kinase inhibits UV- and TNFα-induced JNK and p38 activation.** *A*, Western blotting analyses of HEK293 cells transfected with the indicated plasmids. Cells were lysed at indicated times after incubation with TNFα and analyzed with anti-phospho-JNK (*P-JNK, upper*), -total JNK (*middle*), and -HA (*lower*) antibodies. The immunoblotting analyses were repeated three times. *B*, the procedures are the same as *A*, except the membranes were probed with anti-phospho-p38 (*upper*), -total p38 (*middle*), and -HA (*lower*). Graphical presentations show the normalized density of phosphorylated JNK (*bottom* of *panel A*) and p38 (*bottom* of *panel B*), decaying from 100%.

buffered saline (pH 7.4)). Slides were rinsed with phosphate-buffered saline and incubated in permeabilization solution followed by TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37 °C and then detected with alkaline phosphatase substrate solution (Vector Laboratories, Burlingame, CA) for 10 min at 25 °C. After an additional rinse, the slides were mounted and analyzed under a light microscope. These experiments were performed in triplicate.

RESULTS

AKT2 Is Activated by UV Irradiation, Heat Shock, Hyperosmolarity, and TNFα—Previous studies showed that stress activates AKT1 and AKT3 but not AKT2 in fibroblasts (19). It has also been shown that TNFα receptor mediates UV- and heat shock-induced stress signaling (1–3). In agreement with these studies, we found that exposure of NIH 3T3 fibroblasts to UV-C, heat, or hyperosmotic conditions did not result in AKT2 activation (data not shown). It is possible, however, that stress might activate AKT2 in epithelial cells due to the fact of frequent alterations of AKT2, but not AKT1 and AKT3, in human epithelial tumors (7, 24, 27). For this reason we examined the effects of stress on AKT2 activation in two ovarian epithelial cancer cell lines, A2780 cells, which were transiently transfected with HA-AKT2, and OVCAR3 cells, which express high levels of endogenous AKT2 (7). The cells were exposed to UV-C, heat shock (45 °C), 0.4 M NaCl, or 20 ng/ml TNFα. IGF1-stimulated cells were used as controls. As assessed by *in vitro* kinase and Western blot analyses of AKT2 immunoprecipitates, all the stimuli substantially increased AKT2 activity in both A2780 and OVCAR3 cells (Figs. 1, *A* and *B*). The levels of AKT2 activity induced by these agents, however, were variable. AKT2 activity induced by TNFα and UV was comparable with that stimulated by IGF-1, whereas the effect of heat shock and hyperosmolarity (NaCl) on AKT2 activity was relatively smaller (Fig. 1). Nevertheless, these findings suggest that stresses activate AKT2 in a cell type-specific manner.

Stress Simulates PI3K That Mediates AKT2 Activation—To show that stress does indeed activate PI3K in epithelial cells, A2780 or HEK293 cells were exposed to UV irradiation, heat shock, and 0.4 M NaCl or TNFα, and cell lysates were immunoprecipitated with antibody to pan-p85, a regulatory subunit of PI3K. Assay of PI3K activity shows that these stress conditions as well as TNFα activated PI3K as efficiently as did IGF-1 (Fig. 2*A*). As described above, stress has been shown to activate AKT1 by both PI3K-dependent and -independent pathways

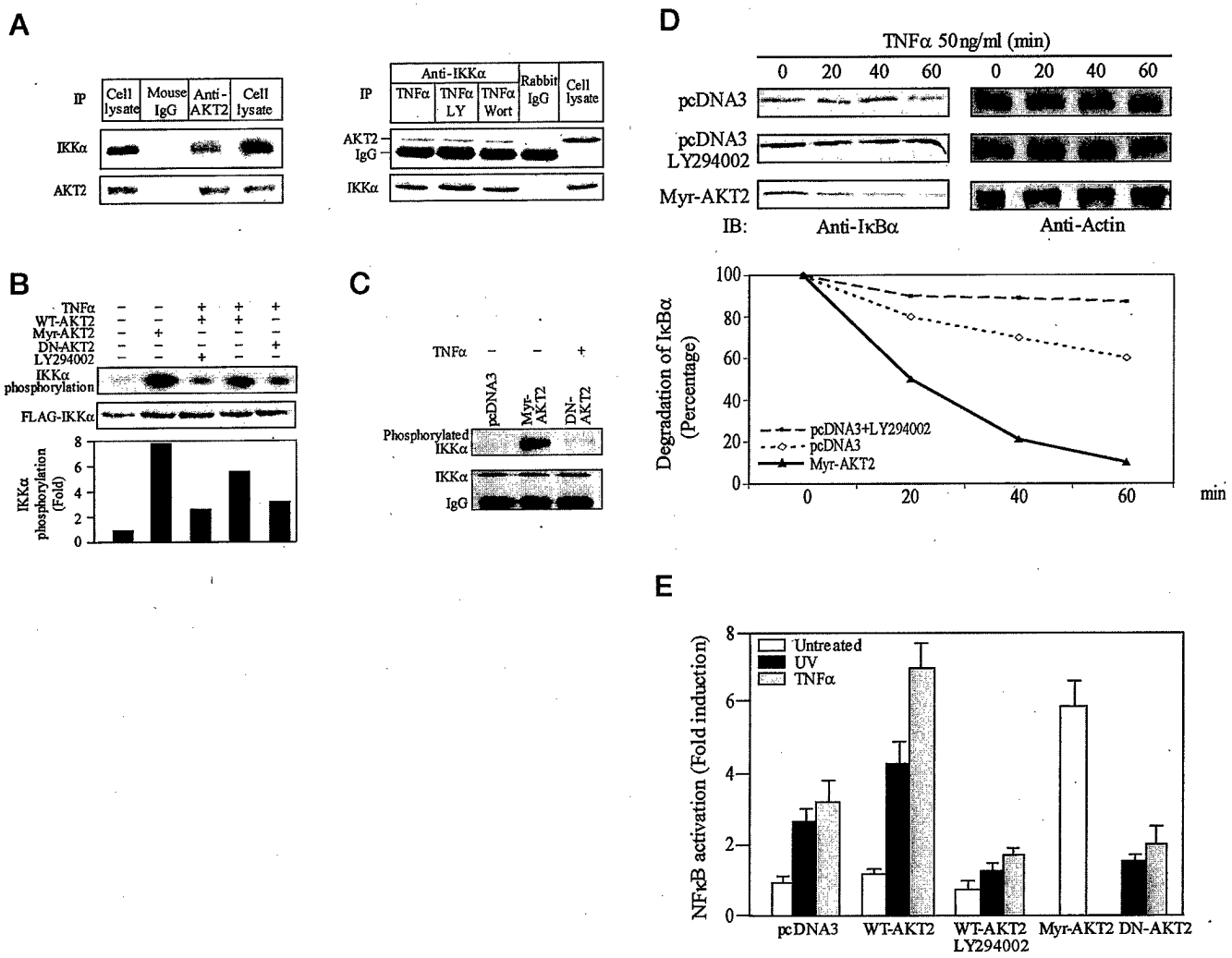


FIG. 4. **AKT2 interacts with and phosphorylates IKKα, leading to IκBα degradation and NFκB activation.** *A*, *left panel*, Western blotting analyses. HEK293 cell lysates were immunoprecipitated (*IP*) with anti-AKT2 or IgG (control) and detected with anti-IKKα (*top*) or anti-AKT2 (*bottom*) antibody. *Right panel*, HEK293 cells were treated with LY294002 (*LY*) or wortmannin for 30 min followed by TNFα for 20 min. Immunoprecipitates were prepared with anti-IKKα antibody or IgG and immunoblotted with antibody to AKT2 (*top*) or IKKα (*bottom*). *B*, *in vitro* kinase assay analyses of immunoprecipitates prepared from A2780 cells transfected with indicated plasmids using immunopurified FLAG-IKKα as substrates (*upper*). Expression of FLAG-IKKα was confirmed by immunoblotting analysis with anti-FLAG antibody (*middle*). The *bottom panel* shows the relative phosphorylation levels of IKKα by AKT2. *C*, *in vivo* labeling of IKKα from COS7 cells transfected with indicated DNA constructs treated with or without TNFα and incubated with [γ-^{32}P]orthophosphate for 4 h. IKKα immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, exposed to film (*top*), and then detected with anti-IKKα antibody (*bottom*). *D*, AKT2 induces IκBα degradation. HEK293 cells were transfected with indicated plasmids and treated with cycloheximide (50 μg/ml) for 1 h before treatment with 50 ng/ml TNFα for up to 60 min. Cell lysates were immunoblotted (*IB*) with antibody to IκBα (*left panels*) or β-actin (*right panels*). Degradation of IκBα was quantified with a densitometer. The *bottom panel* shows the degradation rate of IκBα by normalizing density of IκBα bands at 0 time point as 100%. *E*, reporter assays. HEK293 cells were transfected with 2×NFκB-Luc, β-galactosidase and WT-AKT2, Myr-AKT2, or DN-AKT2 pretreated with or without LY294002 and subsequently exposed to 40 J/m^2 UV-C or 20 ng/ml TNFα. Cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. *Error bars* represent S.D. Data were obtained from triplicate experiments.

(17, 18). To assess the role of PI3K in the stress-induced activation of AKT2, A2780 cells transfected with HA-AKT2 were exposed to 25 μM LY294002, a specific PI3K inhibitor, for 30 min before stress or TNFα treatments. LY294002 effectively inhibited stress- and TNFα-induced AKT2 activation (Fig. 2*B*). These data provide direct evidences of stress-induced activation of AKT2 through a PI3K-dependent pathway in human epithelial cells.

Stress-induced AKT2 Activation Inhibits UV- and TNFα-induced JNK and p38 Activities—Previous studies demonstrated that two groups of mitogen-activated protein kinases, the JNK and p38, are activated by environmental stress and TNFα (28). Therefore, we examined the effects of stress-induced AKT2 activation on the JNK and p38 to determine whether stressed-induced AKT2 activation could target these two stress kinases. A2780 cells were transfected with constitutively active AKT2 or pcDNA3 vector alone. Thirty-six hours after transfection, cells were treated with TNFα or UV and analyzed by Western blot for JNK and p38 activation using anti-phospho-JNK and anti-phospho-p38 antibodies. Both JNK and p38 were activated by TNFα and UV irradiation. The maximal activation was observed at 10 min of stimulation. Expression of constitutively active AKT2, however, exhibited inhibitory effects on the activation of JNK and p38 that was induced by TNFα and UV irradiation. Notably, the activation of JNK and p38 in constitutively active AKT2-transfected cells does not significantly differ from that of the cells transfected with pcDNA3 vector at 10 min of TNFα treatment. However, the phosphorylation levels of JNK and p38 in the cells expressing constitutively active AKT2 declined much more than that of

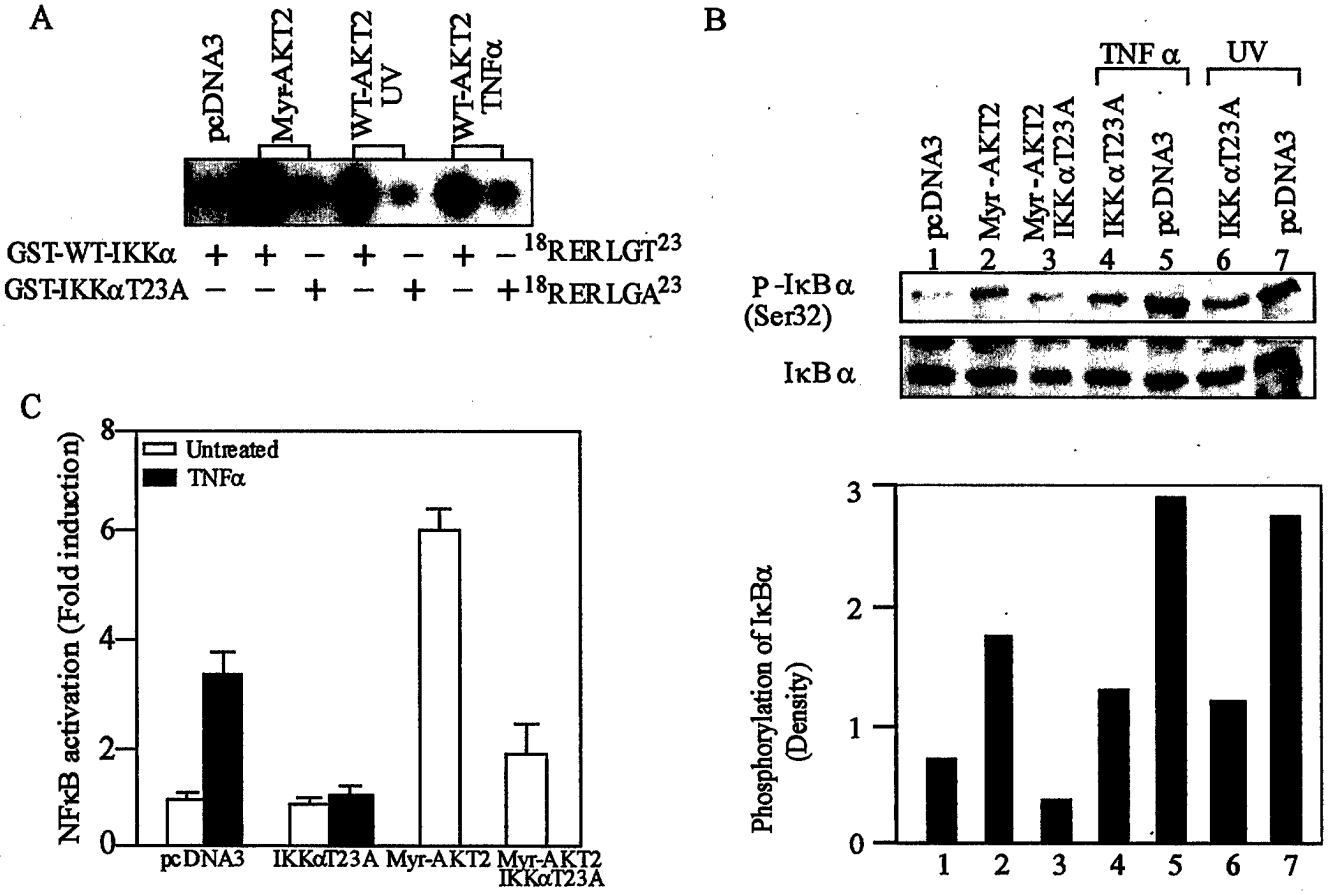


FIG. 5. **AKT2-phosphorylated IKKα at threonine 23 is required for stress-induced NFκB.** *A*, AKT2 phosphorylation of IKKα at threonine 23. *In vitro* kinase assay of AKT2 immunoprecipitates prepared from A2780 cells transfected with the indicated plasmids and treated with or without UV or TNFα. GST-fused wild type IKKα (18**RERLGT**23) or mutant IKKα (18**RERLGA**23) was used as the substrate. *B*, AKT2 induces IκBα phosphorylation (*P-*). HEK293 cells were transfected with the indicated expression constructs. Thirty-six hours after transfection, cells were treated with 20 ng/ml TNFα for 30 min or irradiated with 40 J/m^2 UV followed by a 30-min incubation. Cell lysates were immunoblotted with anti-phospho-IκBα (*upper*) or anti-IκBα (*middle*) antibody. The band density of the phospho-IκBα was quantified (*bottom*). *C*, luciferase reporter assay. HEK293 cells were transfected with the indicated plasmids. After treatment with or without 20 ng/ml TNFα for 12 h, cell lysates were assayed for luciferase activity and normalized by β-galactosidase activity. Results were obtained from three independent experiments.

pcDNA3-transfected cells after 30 min of stimulation (Fig. 3 and data not shown). We therefore conclude that the activation of AKT2 does not activate but rather inhibits TNFα- and UV-induced JNK and p38 activities.

AKT2 Interacts With and Phosphorylates IKKα, but Not NIK, Leading to IκBα Degradation and NFκB Activation—The capacity of both cellular stress and TNFα to activate the NFκB pathway is well documented (29). Previous studies also show that AKT1 induces activation of the NFκB by interaction with IKKα (13, 14). However, to date there are no reports addressing the potential role of AKT2 in the activation of the NFκB pathway. To determine whether AKT2 associates with IKKα, HEK293 cells were treated with or without TNFα, immunoprecipitated with anti-AKT2, and immunoblotted with anti-IKKα antibody or vice versa. In both instances, the association of AKT2 with IKKα was observed (Fig. 4*A*). Additional studies showed that AKT2-IKKα interaction was unaffected by treatment of cells with PI3K inhibitor, wortmannin, or LY294002 (Fig. 4*A*). These findings indicate that AKT2 constitutively associates with IKKα. In addition, we have identified putative AKT2 phosphorylation sites in the IKKα (18**RERLGT**23) and in NFκB-inducing kinase (NIK, 366**RSREPS**371) (bold residue letters represent Akt consensus sequence). To determine whether IKKα and/or NIK are phosphorylated by AKT2, A2780 cells were transfected with different forms of AKT2 and treated with LY294002 and TNFα. *In vitro* AKT2 kinase assays were performed using FLAG-IKKα or HA-NIK, purified from the transfected COS7 cells, as substrate. Repeated experiments show that TNFα-induced AKT2 and constitutively active AKT2 phosphorylated IKKα (Fig. 4*B*) but not NIK (data not shown). Phosphorylation of IKKα induced by TNFα was largely attenuated by PI3K inhibitor LY294002. Quantification analyses revealed that approximate 70% of TNFα-induced IKKα phosphorylation was inhibited by pretreatment with LY294002 (Fig. 4*B*). Furthermore, we assessed AKT2 to determine if it phosphorylates IKKα *in vivo*. COS7 cells were transfected with FLAG-IKKα together with either constitutively active or dominant-negative AKT2 or vector alone and labeled with [γ-^{32}P]orthophosphate. IKKα immunoprecipitates prepared using anti-FLAG antibody were separated by SDS-PAGE and transferred to nitrocellulose. The phospho-IKKα was detected by autoradiography. As shown in Fig. 4*C*, IKKα was highly phosphorylated in cells expressing constitutively active AKT2 but not in the cells transfected with pcDNA3 and dominant-negative AKT2. Collectively, these data indicate that IKKα is an AKT2 physiological substrate.

Activation of NFκB requires its dissociation from its cytosolic inhibitor, IκB, a process dependent on the phosphorylation and consequent degradation of IκB by IKK. Thus, we next examined AKT2 to determine if it induces IκB degradation. Immunoblotting analyses revealed that constitutively active AKT2 significantly promoted IκBα degradation (Fig. 4*D*). To assess the involvement of AKT2 in NFκB activation, HEK293 cells were co-transfected with a NFκB-luciferase reporter and either

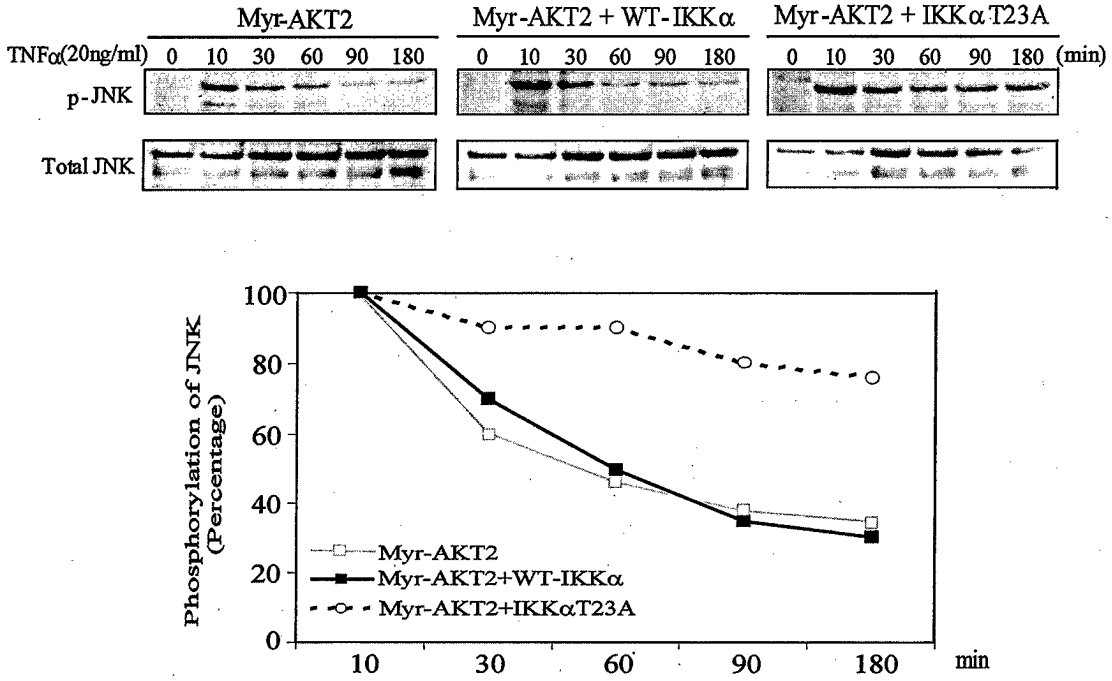


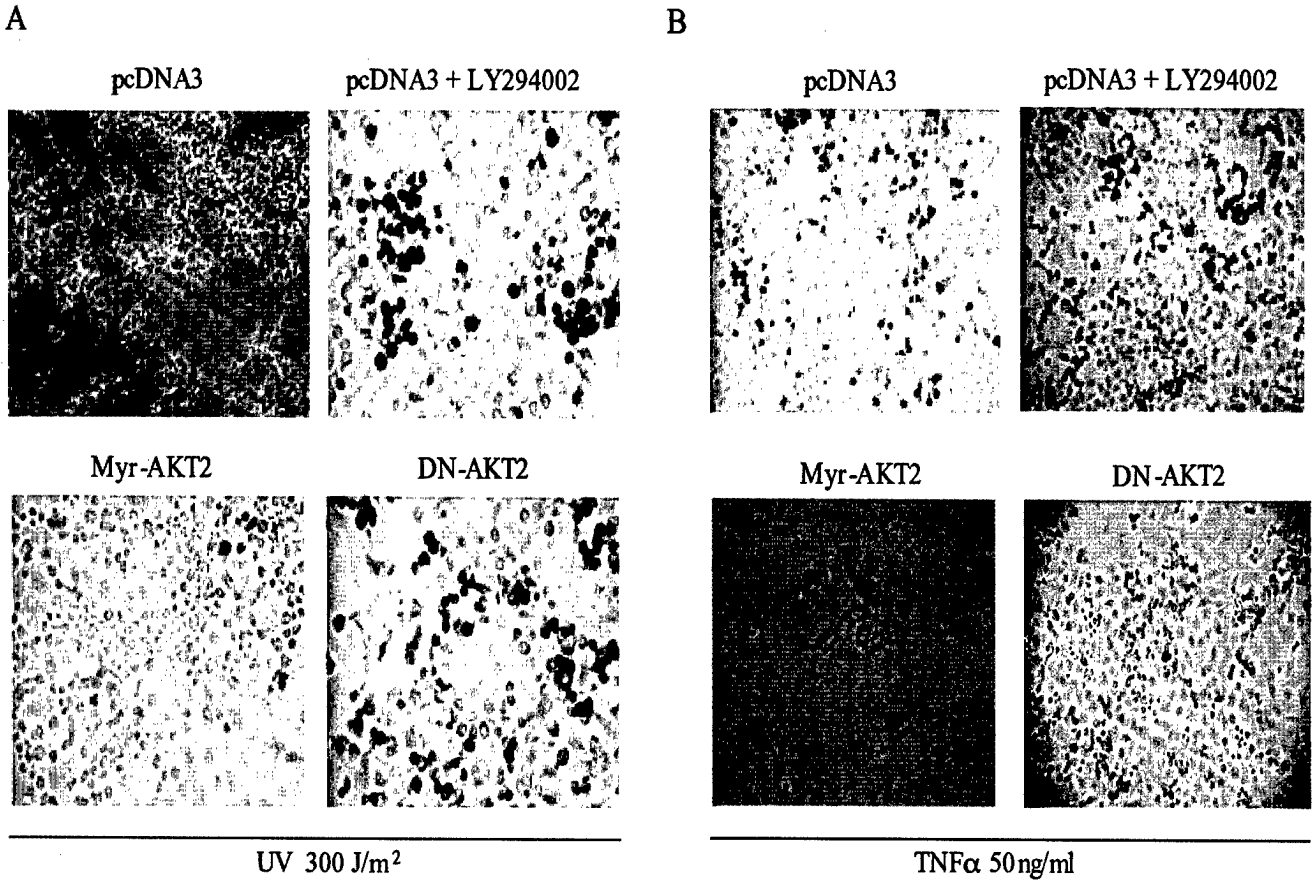
FIG. 6. **AKT2 phosphorylation of IKKα is required for inhibition of TNFα-induced JNK activity.** Immunoblotting analyses of HEK293 cells transfected with indicated expression constructs and treated with TNFα (20 ng/ml). The blots were probed with anti-phospho-JNK (*p-JNK*; *upper*) and -total JNK (*middle*) antibodies. Results represent one of three independent experiments. The *bottom panel* shows the quantification of phosphorylated JNK at the indicated time points.

vector alone, wild type, or constitutively active or dominant negative AKT2 treated with or without LY294002 before UV or TNFα stimulation. As shown in Fig. 4*E*, ectopic expression of wild-type AKT2 significantly enhanced UV- and TNFα-induced NFκB activity, which was abolished by treatment of cells with LY294002 or dominant negative AKT2. Constitutively active AKT2 alone was able to induce NFκB activity to a level comparable with UV- or TNFα-treated cells transfected with wild-type AKT2. These data show that PI3K/AKT2 mediates both stress- and TNFα-activated NFκB pathway.

To determine AKT2 phosphorylation site of IKKα, GST fusion proteins containing either wild type IKKα (^{18}**RERLGT**^{23}; termed GST-WT-IKKα) or mutant IKKα (^{18}**RERLGA**^{23}; termed GST-IKKαT23A) were prepared and used as substrates in *in vitro* AKT2 kinase assays. As seen in Fig. 5*A*, UV- and TNFα-activated AKT2 as well as constitutively active AKT2 phosphorylated GST-WT-IKKα but not GST-IKKαT23A. We next assessed the capacity of AKT2-induced IKKα to phosphorylate IκBα. Constitutively active AKT2 was expressed in HEK293 cells, and cell lysates were immunoblotted with an antibody that specifically recognizes phosphorylated IκBα at Ser^{32}. The results of these experiments show that constitutively active AKT2 increased IκBα phosphorylation ~2-fold and that this increase was abolished by cotransfection of pcDNA3-IKKαT23A. Expression of IKKαT23A also blocked IκBα phosphorylation induced by TNFα or UV (Fig. 5*B*). Additional luciferase reporter experiments demonstrated that expression of IKKαT23A inhibited the TNFα- or constitutively active AKT2-induced NFκB activation (Fig. 5*C*). These data indicate that phosphorylation of IKKα at Thr^{23} is required for AKT2-mediated NFκB activation.

IKKα Phosphorylation by AKT2 Is Required for Inhibition of JNK but Not p38 Activation—Recent studies showed that NFκB exerts its cell survival function by inhibition of JNK activation in response to extracellular stress (30, 31). However, it is currently unknown whether AKT-induced NFκB activation results in inhibition of JNK. Therefore, we next attempted to determine if AKT2-activated IKKα is required for AKT2 inhibition of JNK and p38 activities induced by stress and TNFα. The activation of JNK and p38 was examined in HEK293 cells transfected with IKKα or IKKαT23A together with or without constitutively active AKT2. Western blotting analyses with phospho-JNK and -p38 antibodies revealed that wild type IKKα did not significantly enhance AKT2 inhibition of JNK (Fig. 6). However, expression of IKKαT23A abrogated the effects of constitutively active AKT2 on inhibition of JNK (Fig. 6). Similar to the results shown in Fig. 3, TNFα-induced JNK activation reached the maximal level at 10 min of stimulation, which was neither significantly inhibited by constitutively active AKT2 nor affected by expression of IKKαT23A (Fig. 6). Therefore, these data indicate that inhibition of JNK activation by AKT2/NFκB could be via a mechanism of induction of dephosphorylation of JNK by the AKT2/IKKα/NFκB cascade.

AKT2 Activation Inhibits Stress-induced Apoptosis—It is documented that various stresses and TNFα are capable of inducing apoptosis in different cell types through activation of JNK and p38 pathways (29). Because PI3K/AKT is essential for cell survival and activated AKT2 inhibits JNK/p38 and induces NFκB pathway, we investigated the role of PI3K/AKT2 in stress- and TNFα-induced programmed cell death. AKT2 stably transfected A2780 cells were pretreated with or without LY294002 for 2 h before exposure to UV, heat shock, NaCl, or TNFα. As determined by the TUNEL assay, inhibition of PI3K activity dramatically increased the percentage of cells undergoing apoptosis in response to UV or TNFα (Fig. 7). Moreover, inhibition of AKT2 activity by expression of dominant-negative AKT2 increased the percentage of apoptotic cells in the UV- and TNFα-treated populations by ~2-fold. On the other hand, cells expressing constitutively active AKT2 were resistant to UV- and TNFα-induced apoptosis. These data show that the PI3K/AKT2 pathway plays a key role in protecting cells from apoptosis induced by extracellular stress or TNFα.



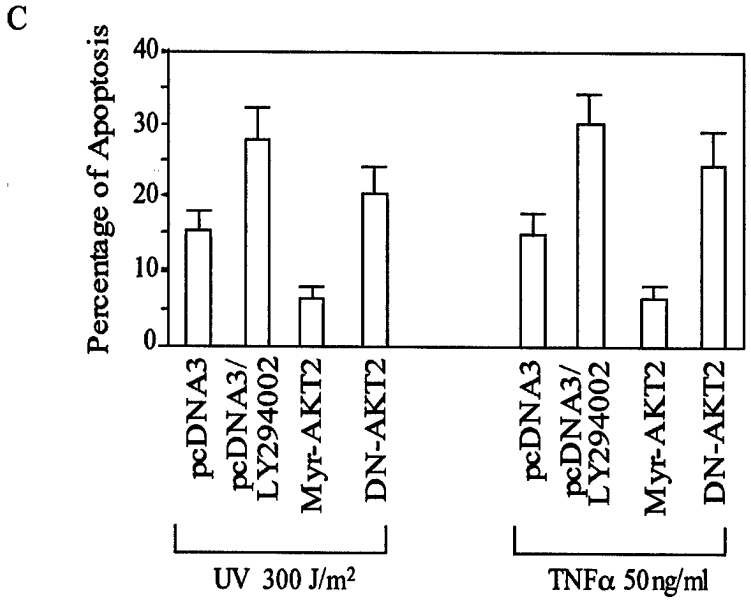


FIG. 7. **AKT2 activation inhibits stress-induced apoptosis.** A2780 cells stably transfected with constitutively active AKT2, DN-AKT2, or vector alone were pretreated with or without 25 μM LY294002 for 2 h before exposure to UV-C (300 J/m²) (*A*) or TNFα (50 ng/ml) for 24 h (*B*). Apoptosis was assessed by TUNEL assay. *C*, quantitation of data shown in *A* and *B* were derived from triplicate experiments. *Error bars* represent S.D.

DISCUSSION

In this report, we have provided evidence that AKT2 is activated by extracellular stress and TNFα through a PI3K-dependent pathway in human epithelial cells. Most importantly, the activation of AKT2 inhibits stress- and TNFα-induced JNK and p38 activities and activates the NFκB cascade, leading to protection of cells from stress- and TNFα-induced apoptosis.

Previous studies show that stress activates cell membrane

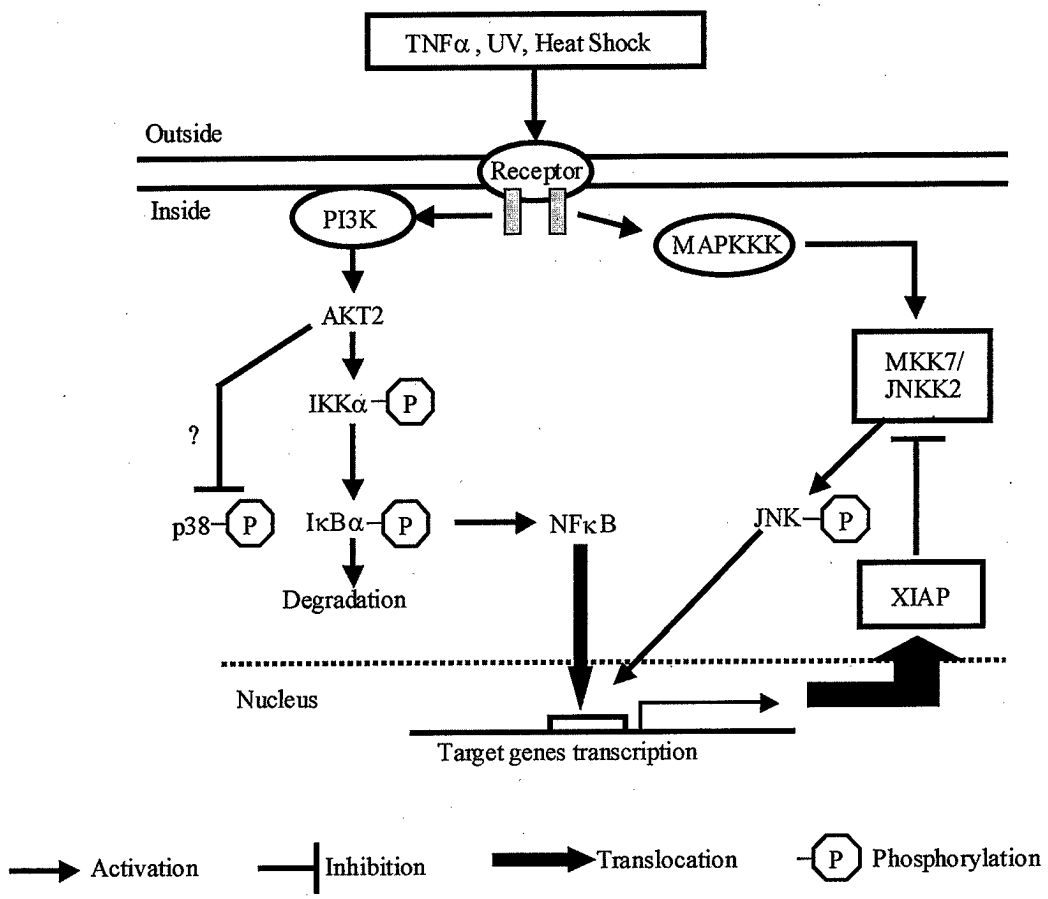


FIG. 8. **Schematic illustration of negative regulation of JNK by AKT2/NFκB.**

receptors, including those for epidermal growth factor, platelet-derived growth factor, and IGF. As a result, receptors associate with numerous proteins that activate downstream signaling molecules (1–3). One such protein is PI3K, which has been implicated in the regulation of nearly all stress signaling pathways (1). Because the AKTs are major downstream targets of PI3K, their role in the stress response has been recently investigated. In Swiss 3T3 cells, both oxidative stress and heat shock were shown to induce a marked activation of AKT1 and AKT3 but not AKT2 (19). AKT1 activation by hyperosmotic stress in COS7 and NIH 3T3 cells has also been demonstrated (17). In this study, we show that AKT2 is activated by different stress conditions including UV irradiation, hyperosmolarity, and heat shock as well as by TNFα in several human epithelial cell lines.

Three isoforms of AKT display high sequence homology and share similar upstream regulators and downstream targets as identified so far. However, there are clear differences between them in terms of biological and physiological function. In addition to the more prominent role of AKT2 in human malignancy and transformation (7, 32), the expression patterns of AKT1, AKT2, and AKT3 in normal adult tissues as well as during development are quite different (4, 8, 33). Recent studies suggest that AKT1, AKT2, and AKT3 may interact with different proteins and, thus, may play different roles in signal transduction. For instance, the Tcl1 oncoprotein preferentially binds to and activates AKT1 but not AKT2 (34). Gene knockout studies revealed that AKT1-deficient mice display defects in both fetal and postnatal growth but, unlike $AKT2^{-/-}$ mice, do not exhibit a type II diabetic phenotype; these differences suggest that the functions of AKT1 and AKT2 are non-redundant with respect to organismic growth and insulin-regulated glucose metabolism (21–23). It has been also shown that AKT2 but not AKT1 plays a specific role in muscle differentiation (35).[2] In this study, we demonstrated that AKT2 is activated by a variety of stress conditions in human epithelial cells but not in fibroblasts, suggesting that activation of different isoforms of AKT is cell type-specific in response to extracellular stress.

It is controversial whether stress-induced AKT1 activation is mediated by the PI3K pathway (17–19). Two previous reports showed that PI3K inhibitors did not block heat shock- or H_2O_2-induced activation of AKT1 and, thus, suggested that stress (unlike growth factors) activates AKT1 in a PI3K-indpendent manner (17, 18). However, the opposite results were observed by other groups (19, 20). Konishi *et al.* also provide evidence of AKT1 activation by H_2O_2 and heat shock through both PI3K-dependent and -independent pathways (18). We previously demonstrated that activation of AKT2 by growth factors required PI3K activity, whereas both PI3K-dependent and -independent pathways contributed to AKT2 activation by Ras (26). In this report, we show that PI3K inhibitors completely block AKT2 activation induced by UV-C, heat shock, and hyperosmolarity, indicating that stress activates AKT2 via the PI3K pathway.

JNK and p38 are stress mitogen-activated protein kinases that are activated by cytokines and a variety of cellular stresses (28). Like the classical mitogen-activated protein kinase kinase (MEK), direct activators for JNK and p38 have been identified. JNK is activated by phosphorylation of tyrosine and threonine by the dual specificity kinases, MKK4/SEK1 and MKK7. Similarly, p38 is activated by MKK3 and MKK6. However, biochemical studies have documented the existence of other JNK

[2] S. Kaneko, S. V. Nicosia, Z. Wu, T. Nobori, and J. Q. Cheng, submitted for publication.

and p38 activators or inhibitors in cells stimulated by a variety of cellular stresses (28). Although previous reports showed that AKT, JNK, and p38 are downstream targets of PI3K and represent parallel pathways in response to stress (17–20, 37, 38), the data presented in this study indicate that stress- and TNFα-induced activation of AKT2 inhibits the JNK and p38 activities, suggesting that AKT2 cross-talks with JNK and p38 stress pathways.

NFκB is another critical stress response pathway (29). Activation of NFκB is achieved through the signal-induced proteolytic degradation of IκB, which is associated with and inhibits the activity of NFκB in the cytoplasm. The critical event that initiates IκB degradation is the stimulus-dependent activation of the IκB kinases IKKα and IKKβ, which phosphorylate IκB at specific N-terminal serine residues (Ser^{32} and Ser^{36} for IκBα; Ser^{19} and Ser^{23} for IκBβ). Phosphorylated IκB is then selectively ubiquitinated by an E3 ubiquitin ligase and degraded by the 26 S proteasome, thereby releasing NFκB for translocation to the nucleus where it initiates the transcription of target genes (29). Moreover, two mitogen-activated protein kinase kinase kinase (MAPKKK) members, NIK and MEKK1, have been reported to enhance the activity of the IKKs and consequently trigger the phosphorylation and destruction of the IκBs and induce the activation of the NFκB pathway (29). Recent studies also showed that AKT1 induces the NFκB cascade through activation of IKK and degradation of IκB (13, 14). In this report, we show that AKT2 physically binds to and phosphorylates IKKα but not NIK even though NIK contains an AKT2 phosphorylation consensus sequence. When activated by stress or TNFα, AKT2 degrades IκB and activates NFκB-mediated transcription, indicating that stress-activated AKT2 targets the NFκB pathway.

Importantly, we have provided evidence that activation of AKT2 induced by stress and TNFα inhibits JNK activity through activation of the NFκB pathway to protect cells from apoptosis in response to these stimuli. Previous studies showed that the AKT2 pathway is important for cell survival and malignant transformation (7, 24, 32). The data presented here show that cells expressing constitutively active AKT2 are resistant to stress- and TNFα-induced apoptosis and that dominant-negative AKT2 and LY294002 sensitize cells to stress- and TNFα-induced programmed cell death. These findings indicate that stress-induced AKT2 activation promotes cell survival. Among the stress-activated kinases are JNK; recent studies demonstrated that activation of JNK and p38 plays an important role in triggering apoptosis in response to extracellular stress and TNFα (36, 39–41), whereas activation of NFκB protects cells from programmed cell death (29). Although a number of downstream targets of AKT2 have been identified, our data indicate that AKT2-inhibited JNK and p38 activities and AKT2-induced NFκB activation could play, at least in part, an important role in the AKT2 pathway that protects cells from stress- and TNFα-induced apoptosis. Recent reports demonstrate that NFκB-up-regulated Gadd45β and Xiap inhibited JNK activation and abrogated TNFα-induced programmed cell death (30, 31). Our cDNA microarray experiments showed that constitutively active AKT2 induces *Xiap*.[3] Thus, AKT2 inhibition of JNK activity could be due to up-regulation of Xiap by NFκB pathway (Fig. 8). Further studies are required to characterize the mechanism of inhibition of p38 stress pathway by AKT2 and involvement of Xiap in AKT2/NFκB inhibition of the JNK activation.

[3] M. Sun and J. Q. Cheng, unpublished data.

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Positive Feedback Regulation between Akt2 and MyoD during Muscle Differentiation

CLONING OF *Akt2* PROMOTER*

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Akt2 is a member of the Akt/PKB family, which is involved in a variety of cellular events including cell survival, proliferation, and differentiation. During skeletal muscle differentiation, the Akt2 but not Akt1 expression was significantly increased. Microinjection of anti-Akt2 but not anti-Akt1 antibody efficiently abrogated myogenesis, indicating that Akt2 plays a specific role in muscle differentiation. It has been well documented that ectopic expression of MyoD is sufficient to induce muscle differentiation in myoblasts. However, the mechanism of induction of Akt2 during muscle differentiation and the significance of Akt2 protein in MyoD-induced myogenesis are largely unknown. In this study, we provide direct evidence that *Akt2* is transcriptionally regulated by MyoD and activates MyoD-myocyte enhancer binding factor-2 (MEF2) transactivation activity. The *Akt2* promoter was isolated and found to contain nine putative E-boxes (CANNTG), which are putative MyoD binding sites. Electrophoretic mobility shift analyses revealed that MyoD bound to eight of the sites. The expression of MyoD significantly enhanced *Akt2* promoter activity and up-regulated Akt2 mRNA and protein levels. Moreover, Akt2 but not Akt1 was activated during differentiation. The expression of Akt2 activated MyoD-MEF2 transcriptional activity and induced myogenin expression. These data indicate that there is a positive feedback regulation loop between Akt2 and MyoD-MEF2 during muscle differentiation, which is essential for MyoD-induced myogenesis.

Skeletal muscle differentiation requires an ordered multiple step process in which myoblasts irreversibly exit from the cell cycle, elongate, and fuse into multinucleated myotubes. This program is driven by the expression of the MyoD family of transcription factors and the myocyte enhancer binding factor-2 (MEF2)[1] family members (1). The MyoD family (also called myogenic regulatory factors) of basic helix-loop-helix proteins includes MyoD, myogenin, Myf5, and myogenic regulatory factor-4. Forced expression of MyoD transcription factor can inhibit cell cycle progression and induce muscle differentiation. The transcription of many muscle-specific genes is activated by the binding of MyoD factors to a simple consensus sequence of CANNTG termed an E-box, present in regulatory regions of many muscle-specific genes. The MEF2 family of transcription factors includes MEF2A, MEF2B, MEF2C, and MEF2D, which belongs to the MADS (MCM1, agamous, deficiens, serum response factor) box transcription factors. Evidence indicates that the members of MyoD and MEF2 families interact with each other to synergistically induce muscle-restricted target genes (2). One of the targets is the gene encoding myogenin, which is one of the earliest molecular markers for myoblasts committed to differentiation. The up-regulation of myogenin in concomitant with the induction of the cyclin-dependent protein kinase inhibitor $p21^{Waf/Cip1}$ indicates that the cells have irreversibly exited from the cell cycle and entered the differentiation program (3).

Unlike most growth factors that stimulate myoblast proliferation and inhibit muscle differentiation, the insulin-like growth factors (IGF-I and IGF-II) are potent stimulators of muscle differentiation through induction of myogenin and MEF2 (4). However, the intracellular myogenic signaling process dependent on IGFs is poorly understood. Studies on signaling through IGF receptors have revealed two main pathways, MAPK and PI3K, by which these signals might be transmitted. Several reports showed that the PI3K inhibitors (LY294002 and wortmannin) and a dominant negative p85α (the PI3K regulatory subunit) block IGF-induced myogenesis, whereas the MAPK inhibitor PD098059 enhanced IGF-stimulated muscle differentiation (5–7). Moreover, recent studies demonstrate that constitutively activated PI3K enhanced the transcriptional activity of both MyoD and MEF2 (6, 8). Taken collectively, these studies strongly indicate the essential role of PI3K in myogenesis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF452411.

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[1] The abbreviations used are: MEF2, myocyte enhancer binding factor-2; MAPK, mitogen-activated protein kinase; PKB, protein kinase B; HEK, human embryonic kidney; Luc, luciferase; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP-response element-binding protein; HDAC, histone deacetylase; PI3K, phosphatidylinositol 3-kinase; IGF, insulin-like growth factor.

The serine/threonine protein kinase Akt (also named PKB) is a major downstream target of PI3K and has been implicated in muscle differentiation (9). Three different isoforms of Akt have been identified including Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, all of which are activated by growth factors in a PI3K-dependent manner. The full activation of the Akt requires phosphorylation at Thr^{308} (Akt1), Thr^{309} (Akt2), or Thr^{305} (Akt3) in the activation loop and Ser^{473} (Akt1), Ser^{474} (Akt2), or Ser^{472} (Akt3) in the C-terminal activation domain (10). The most studied isoform is Akt1, which mediates IGF signaling to regulate cell survival, cell growth, GLUT4 translocation, and muscle differentiation. It has been shown that ectopic expression of constitutively activated Akt1 can promote extensive differentiation in different myoblast cell lines in the absence of IGF-I and can reverse the inhibitory effects of PI3K inhibitors LY294002 and wortmannin on myogenic differentiation (5, 9, 11). However, several studies including ours show that both the mRNA and protein levels of the endogenous Akt1 were not changed, whereas Akt2 was elevated during muscle differentiation, suggesting that Akt2 but not Akt1 plays a specific role in myogenesis under physiological condition (12–14). A recent study provides compelling supporting evidence by showing that microinjection of Akt2 antibody inhibited the differentiation of muscle cells, whereas anti-Akt1 antibody did not inhibit cell differentiation (15). However, the mechanism by which Akt2 is involved in myogenesis is currently unknown. In this study, we cloned the *Akt2* promoter and demonstrated that MyoD transcriptionally regulates AKT2. During muscle differentiation, elevated Akt2 in turn activated MyoD-MEF2 transactivation activity resulting in myogenin expression.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Materials—Human epithelial kidney (HEK)293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. C2C12 mouse myoblasts were grown in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (growth medium). To induce differentiation, C2C12 cells were maintained in Dulbecco's modified Eagle's medium containing 10% horse serum (differentiation medium). pCSA-MyoD was kindly provided by Dr. Lassar (Harvard Medical School, Boston, MA). The FLAG-tagged MyoD was constructed by subcloning MyoD into p3X-FLAG-CMV10 (Sigma). MEF2 and myogenin-Luc plasmids are described elsewhere (6). The antibodies to Akt1 and Akt2 were purchased from Upstate Biotechnology, and anti- MyoD, myogenin, actin, and FLAG antibodies were from Santa Cruz Biotechnology.

Transcription Start Site Mapping of Human AKT2 Gene—For the analysis of the Akt2 transcription start site, human OVCAR3 mRNA was reverse-transcribed at 55 °C using Superscript reverse transcriptase (Invitrogen) and an Akt2 exon 1-specific reverse complement oligonucleotide 5′-TTCTTTGATGACAGACACCTCATT-3′. Synthesized cDNAs were amplified by polymerase chain reaction using a series of forward primers specific for the DNA sequences within the 8,500 bp upstream of the translation start site and a reverse primer from the coding region of exon 1 (GenBank™ accession number AF452411), and the products of these reactions were resolved by agarose gel electrophoresis.

Cloning and Analysis of the Human Akt2 Promoter—For the reporter analysis of the *Akt2* promoter, DNA fragments containing *Akt2* genomic sequences were amplified from a cosmid clone (pWE9–3), which was obtained by the screening of a human placenta genomic library (Stratagene) with 5′ sequence of *Akt2* cDNA using the polymerase chain reaction and primers derived from human genomic *Akt2* (GenBank™ accession number NT011250). Amplified DNA fragments were subcloned into the luciferase reporter vector pGL3 (Promega). The integrity of all constructs was confirmed by DNA sequencing. Luciferase assays were performed using the luciferase assay system (Promega), and activities were normalized to β-galactosidase activity.

Northern and Western Blot Analysis—Northern blot analysis of total cellular RNA was performed according to standard procedures. Hybridized ^{32}P-labeled probes were visualized and quantified using PhosphorImager analysis (Molecular Dynamics). Western blot analysis was performed as described previously (12).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Analysis—The nuclear extracts were prepared from the FLAG-tagged MyoD-transfected HEK293 cells following the procedures described previously (16). Normalized extracts containing 3–8 μg of protein were incubated with ^{32}P-radiolabeled double-stranded oligonucleotide probes containing each of the nine MyoD binding sequences: 5′-GTCTCAAGTGAGGCAGGTGGTGAGCCCATAA-3′ (−1861/−1832); 5′-GTGCAAGGGGTTCCACCTGCTGCCCCTCCC-3′ (−1750/−1722); 5′-GCCCCGGCTCTCTCACCTGCTTCCGGTTTTG-3′ (−1512/−1483); 5′-GAGGCAAGTGCTGTGAGGGGAAGGGAACGCT-3′ (−982/−953); 5′-GCGGGGCCAAGGCAGATGGCCCCTGGCAGC-3′ (−754/−724); 5′-GCCTCCAGCCCCACCTGCTCCAAGCAGA-3′ (−432/−403); 5′-GCTCCAAGCAGACAGATGGGGCGAGTGTAG-3′ (−415/−386); 5′-GTAGGTAAAAATTCACCTGTTCAGAAACATA-3′ (−260/−231); and 5′-GTAGCTGGAATTACAGGTGCCTGCCACCAG-3′ (−56/−22). Protein-DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

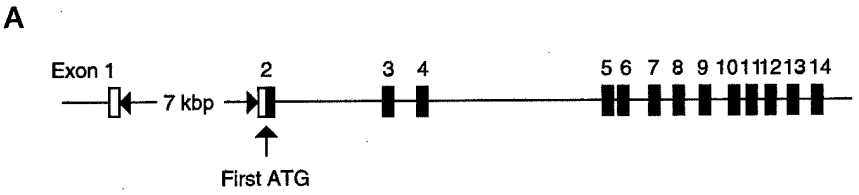
Competition and Supershift Controls for Electrophoretic Mobility Shift Analysis—For competition controls, nuclear extracts were incubated with radiolabeled probes in the presence of 100-fold molar excess unlabeled competitor probe prior to PAGE. For supershift assay, 1 μl of anti-FLAG antibody was incubated with nuclear extracts for 20 min at room temperature prior to the addition of radiolabeled probe and PAGE.

RESULTS

Akt2 Promoter Contains Nine MyoD Binding Elements—To analyze the transcriptional regulation of the serine/threonine protein kinase *Akt2*, we cloned the 5′-flanking region of Akt2 gene from a pWE-15 cosmid human placenta genomic library using the 5′-non-coding region of *Akt2* cDNA as probe. Three overlapping cosmid clones were obtained. Sequence analyses revealed that the Akt2 gene consists of 14 exons. Exon 1 is an untranslated region, and the first intron is >7.6 kilobases in length. The translation initiation site, ATG, of Akt2 protein resides within the exon 2 (Fig. 1*A*). The transcription start site, which was determined by 5′-RACE PCR, lies 7,829 bp upstream of the translation start site. Transcription element analyses of the 2,000 bases of upstream of the transcription start site of the *Akt2* gene, which is considered the putative Akt2 promoter, revealed multiple binding sites for MyoD, Oct1, and p300 and single sites for AP1, C/EBPβ, C/EBP, CREB, and SP1 (Fig. 1*B*). The transcription factor that has the most binding sites in Akt2 promoter is MyoD (nine putative MyoD binding sites: −1852/−1841, −1741/−1731, −1502/−1493, −981/−972, −759/−736, −422/−413, −405/−396, −250/−242, and −41/−32). A MyoD binding site is also called an E-box and its consensus sequence is CANNTG (Fig. 1*B*).

Defining the MyoD Binding Site(s) in the Akt2 Promoter—To determine the MyoD binding elements, we carried out the electrophoretic mobility shift analysis. Nine double-stranded oligonucleotides, each containing an E-box from the *Akt2* promoter, were labeled with ^{32}P and incubated with the nuclear extract from FLAG-tagged MyoD-transfected HEK293 cells. The quality of the nuclear extracts was examined with oligonucleotides derived from an E-box of MEF-2 (data not shown). Mobility shift was observed in −1861/−1832, −1750/−1722, −1512/−1483, −754/−726, −432/−403, and −415/−386, −260/−231, and −51/−22 fragments (Fig. 1*C*). The formation of the electrophoretically retarded complexes was inhibited when an excess of unlabeled oligonucleotides (competitor) were introduced (*middle lane* of each E-box). Moreover, an addition of an anti-FLAG antibody to the reaction mixtures induced the supershift of the protein-DNA complexes appearing in −1861/−1832, −1750/−1722, −1512/−1483, −754/−726, −432/−403, −415/−386, −260/−231, and −51/−22 (Fig. 1*C*). These results indicate that eight of the *Akt2* protomer-derived E-box oligonucleotides can specifically bind MyoD.

MyoD Transactivates the Akt2 Promoter—To investigate whether MyoD regulates the transcription of *Akt2*, a 3.1-kilobase genomic fragment corresponding to the region from bases −2898 to +220 containing nine putative MyoD binding sites,



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TTGAGCAGTTGCTCTTACAGGCTCTTTTGGGAGGACTTCATGTGGATTAACTCACTGAACCCTCCAATAGCCTTAGAG 220
```

MyoD, p300, Oct1, AP1, MyoD, Oct1, MyoD, Sp1, MyoD, CREB, CCAAT, C/EBP, MyoD, MyoD, p300, MyoD, Oct1, C/EBP, MyoD

C

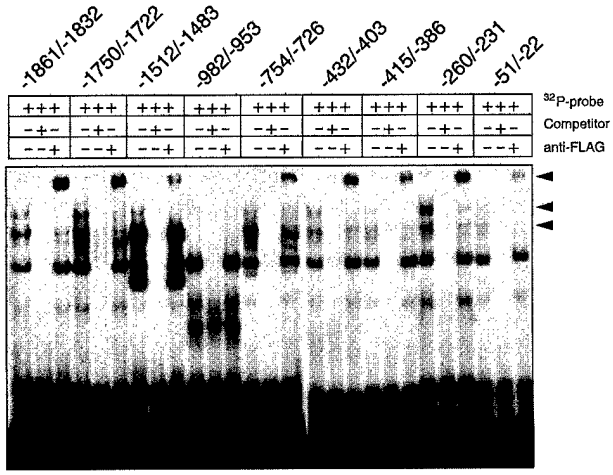

FIG. 1. **Human *Akt2* promoter contains multiple MyoD binding sites.** *A*, schematic representation of the human *Akt2* genomic locus. The exons are shown as *boxes 1–14*. *B*, *Akt2* promoter sequence. Putative transcription factor binding sites are *boxed*. *C*, MyoD binding to the DNA element from the *Akt2* promoter. The electrophoretic mobility shift analysis of double-stranded oligonucleotides containing each MyoD binding sites as indicated at the top. Equal amounts of ^{32}P-labeled oligonucleotides were incubated with nuclear extract prepared from FLAG-MyoD-transfected HEK293 cells in the presence or absence of a 100-molar excess of the unlabeled oligonucleotides (competitor). Supershift was examined by incubation of the reactions with anti-FLAG antibody.

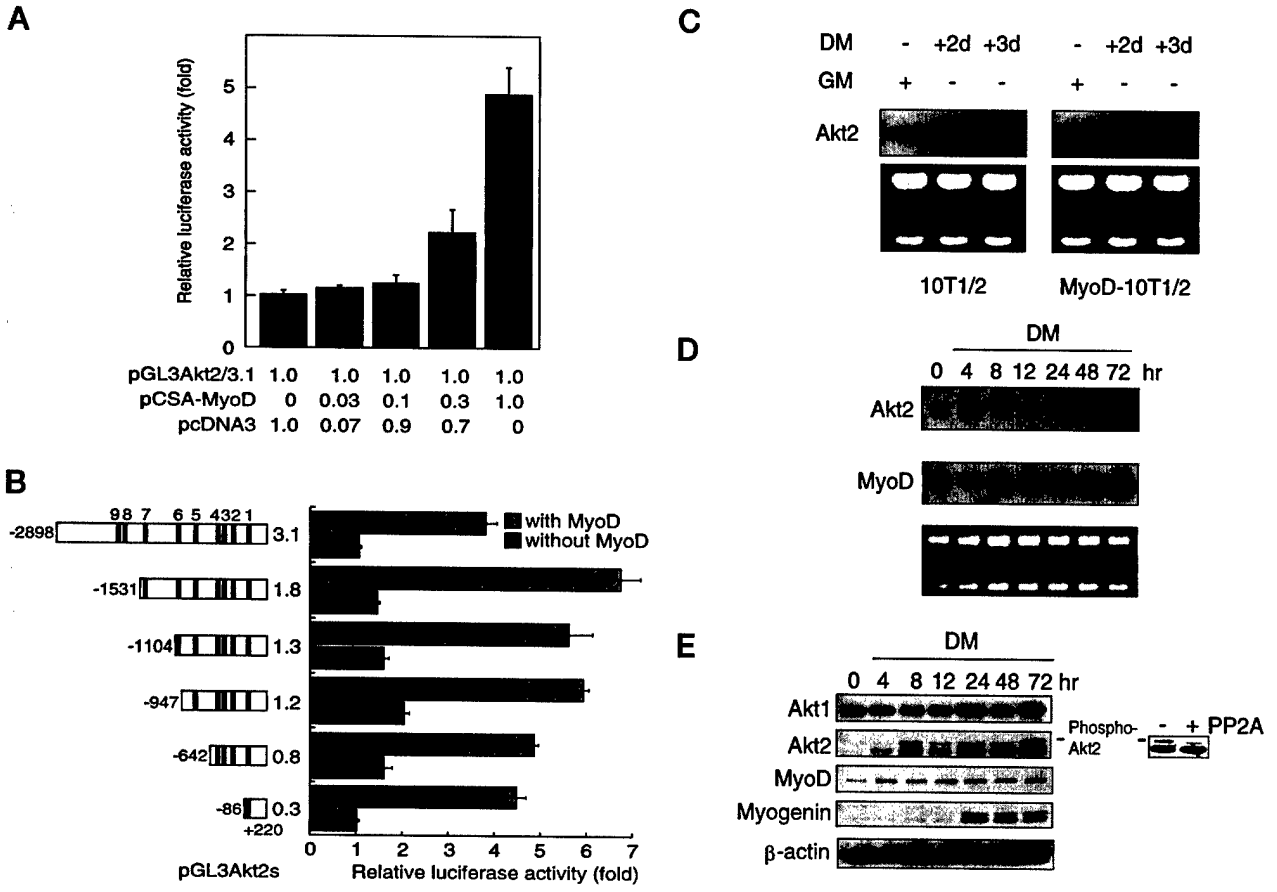

FIG. 2. **MyoD transactivates the *Akt2* promoter and induces Akt2 expression.** *A*, MyoD-induced Akt2 promoter activity occurs in a dose-dependent manner. HEK293 cells were transfected with different amounts of MyoD and pGL3Akt2/3.1 reporter, which contains all nine E-boxes in the presence of tracer amounts of β-galactosidase. Luciferase activity in cell lysates at 36 h posttransfection was normalized to galactosidase and expressed relative to values obtained using the empty reporter plasmid. Results shown are the mean ± S.E. of three independent experiments performed in triplicate. *B*, the first MyoD binding site (−51/−22) is sufficient to activate the Akt2 promoter. HEK293 cells were transfected with the indicated different lengths of Akt2 reporters together with or without MyoD. *C*, Akt2 mRNA is induced in MyoD-transfected but not parental 10T1/2 cells. The cells were cultured in growth medium (*GM*) for 24 h (1 day) and then switched to differentiation medium for 48 (*+2d*) and 72 h (*+3d*). Total RNAs (20 μg) were subjected to Northern blot analysis with [^{32}P]dCTP-labeled Akt2 probe. *D* and *E*, Akt2 is elevated at mRNA and protein levels and activated during C2C12 cell differentiation. The C2C12 cells were cultured in differentiation medium (growth medium) for the indicated times. RNA and cell lysates were analyzed by Northern and Western blots with indicated isotope-labeled probes and antibodies, respectively. Phosphorylated Akt2 migrated slower and was abrogated by PP2A phosphatase treatment (*E*, panel 2).

was subcloned upstream of the luciferase gene in pGL3 basic vector (pGL3-AKT2/3.1). A co-transfection of pGL3-Akt2/3.1 with MyoD into HEK293 cells resulted in a significant increase in reporter activity compared with the control sample co-transfected with the reporter and an empty vector (pcDNA3). Moreover, *Akt2* promoter is regulated by MyoD in a dose-dependent manner (Fig. 2*A*). A similar level of induction of Akt2 reporter activity was also observed upon the transfection of 10T1/2 cells, which lack the endogenous MyoD (data not shown).

To define the MyoD-responsive regions in this promoter, we constructed a group of deletion reporters containing the Akt2 promoter serially deleted from the 5′ end of the −2898 to +220 fragment (Fig. 2*B*). The deletion of −2898 to −1531 significantly increased the MyoD responsiveness by ~40%, even though two potential myoD binding sites were eliminated, suggesting the presence of inhibitory elements for MyoD responsiveness within this region. The further deletion of E-box 7 reduced MyoD responsiveness by 15%. The deletion of the region from −642 to −86 (pGL3-AKT2–0.3), containing a cluster of three E-boxes, deceased the MyoD responsiveness by ~16%. Nevertheless, pGL3-AKT2–0.3, which contains only an E-box, was still induced by MyoD >4.5-fold (Fig. 2*B*), suggesting that the E-box 1 could be a major MyoD response site within the promoter.

Akt2 Is Induced by MyoD during the Muscle Differentiation—We next examined whether MyoD induces mRNA of *Akt2*. Because 10T1/2 myoblast do not express MyoD and are unable to differentiate to myotubes, we have established a 10T1/2 cell line, which was stably transformed with a *MyoD* expression vector. These *MyoD*-transformed cells expressing myocyte-specific markers form multinucleated myotubes when exposed to mitogen-poor differentiation medium (17, 18). The levels of *Akt2* mRNA were evaluated in parental and MyoD-transfected 10T1/2 cells in both growth medium and differentiation medium. *Akt2* mRNA was significantly increased in the 10T1/2-MyoD cells, but this induction did not occur in the parental 10T1/2 fibroblasts when exposed to the differentiation culture medium (Fig. 2*C*).

We further investigated the status of Akt1 and Akt2 in C2C12 cells, which express endogenous MyoD, during differentiation. Western immunoblot analysis revealed that Akt1 protein is stably expressed at a relative high level prior to and during differentiation (Fig. 2*E*). However, both mRNA and protein levels of Akt2 were very low in C2C12 myoblasts cultured in high mitogen growth medium but progressively increased following exposure of cultures to differentiation medium (Fig. 2, *D* and *E*). Moreover, Akt2 kinase activity was induced after switching the culture to differentiation medium

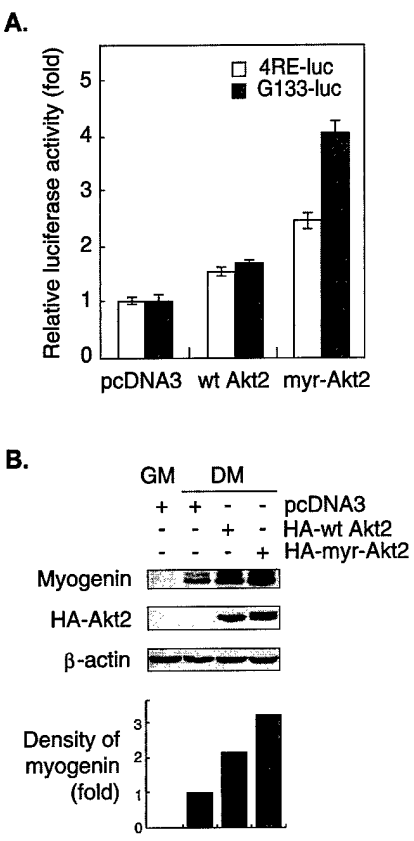

FIG. 3. **Akt2 activates the MyoD and MEF2 transactivation activities and induces myogenin expression.** *A*, wild type and constitutively active Akt2 induce MyoD- and MEF2-dependent reporter activity. C2C12 cells were transfected with different forms of Akt2 along with either 4RE-Luc, which contains four MyoD binding sites, or G133-Luc having both MEF2 and MyoD binding sites. After 24 h of the transfection and 16 h of incubation in differentiation medium, cell lysates were subjected to luciferase assay analysis. *B*, C2C12 cells were transfected with indicated plasmids, cultured in growth medium for 24 h, and then replaced with differentiation medium for 16 h. The expression of myogenin and transfected Akt2 was analyzed by Western blot analysis.

(Fig. 2*E*), suggesting an important role for Akt2 in myogenesis.

Akt2 Induces Myogenin and Activates MyoD-dependent Reporter Genes—The induction of mRNA/protein and kinase activity of Akt2 during muscle differentiation suggests that it regulates muscle-specific gene(s) that controls differentiation. In fact, a previous report shows that ectopical expression of Akt1 and Akt2 could induce muscle-specific gene muscle creatine kinase, and Akt2 was more effective than Akt1 (15). However, the mechanism of Akt induction of muscle-specific gene expression has not been well documented. To explore this hypothesis further, we tested the effects of Akt2 on myogenin expression and MyoD transcriptional activity. G133-Luc, which is a 133-bp myogenin proximal promoter containing MyoD and MEF2 binding sites or 4RE-Luc, which is a MyoD-dependent reporter gene containing four MyoD binding sites, was co-transfected into C2C12 myoblasts with either wild type Akt2 or constitutively active Akt2. The expression of the wild type Akt2 induced G133-Luc and 4RE-Luc reporter activities at 1.6- and 1.5-folds, respectively, whereas the levels of G133-Luc and 4RE-Luc reporter activities were significantly increased (4.2- and 2.5-fold) in the cells transfected with the constitutively active Akt2 (Fig. 3*A*). Consistent with the reporter results, myogenin expression was induced by both wild type and constitutively active Akt2 in C2C12 cells after 16 h of exposure to differentiation medium (Fig. 3*B*). These data suggest that

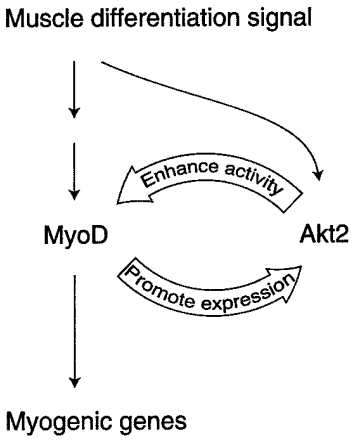

FIG. 4. **Schematic illustration of positive feedback regulation between Akt2 and MyoD during the muscle differentiation.**

AKT2 can up-regulate the endogenous myogenin expression and promote the MyoD transcriptional activity during muscle differentiation.

DISCUSSION

Previous studies demonstrate that IGF1-induced muscle differentiation was primarily mediated by the PI3K/Akt pathway (5–9). Among the three isoforms of Akt family, Akt2 is highly expressed in skeletal muscle (12) and plays a specific role in muscle differentiation as demonstrated by up-regulation of Akt2 but not Akt1 and Akt3 during differentiation and abrogation of myotube formation by anti-Akt2 antibody (15). However, the mechanisms by which Akt2 is up-regulated during the muscle differentiation and stimulates myotube formation are currently unclear. In this report, we provide evidence showing that Akt2 promoter possesses multiple MyoD binding sites, that the expression of Akt2 was induced by MyoD through stimulation of its promoter activity, and that the elevated Akt2 activated MyoD transactivation and induced muscle-specific gene myogenin expression to trigger muscle cell differentiation. Our data indicate a positive feedback regulation loop between Akt2 and MyoD during skeletal muscle differentiation (Fig. 4).

In ectopic expression systems, three isoforms of Akt display very similar functions including muscle differentiation. In fact, previous studies have mostly focused on Akt1 and demonstrated that Akt1 is a critical intermediate in IGF1-induced muscle differentiation hypertrophy and muscle survival (5–9, 19). A previous study shows that the expression of constitutively activated Akt1 induces the transactivation activity of MyoD and MEF-2 (6). However, under physiological conditions, Akt2 seems to play more important roles in myogenesis, because mRNA and protein levels of Akt2 but not Akt1 were up-regulated during muscle differentiation (Fig. 2). In addition, accumulated studies have shown clear differences between these three isoforms in terms of biological function. (*a*) *Akt1* expression is relatively uniform in various normal organs, whereas high levels of *Akt2* are detected in skeletal muscle and heart (12, 20, 21). (*b*) The inhibition of Akt2 but not Akt1 expression abrogates IGF1-induced muscle differentiation (15). (*c*) *Akt2* and *Akt3* but not *Akt1* are amplified and/or up-regulated in certain types of human cancer (22). (*d*) NIH3T3 cells are transformed by wild type Akt2 but not Akt1 and Akt3 (23). (*e*) *Akt2*- and *Akt1*-deficient mice displayed different phenotypes. *Akt2* knock-out mice exhibited a typical type 2 diabetic phenotype that cannot be compensated by the presence of *Akt1* and *Akt3* (24). In contrast, Akt1$^{-/-}$ mice exhibited no diabetic phenotype (25, 26) but showed an impairment in organismal growth, *i.e.* smaller when compared with wild type littermates.

Such relatively subtle phenotype of Akt1$^{-/-}$ mice suggests that Akt2 and Akt3 may substitute to some extent for Akt1 (25). Nevertheless, these data indicate that there are non-redundant functions between three isoforms of Akt in certain tissue and/or cell types. In this study, we cloned *Akt2* promoter and identified multiple MyoD binding sites in *Akt2* (Fig. 1) but not in *Akt1* promoter,[2] further indicating the different transcriptional regulation between *Akt1* and *Akt2*.

Previous studies have shown that MyoD and MEF2 transcription factors interact with each other to synergistically induce muscle-restricted target gene expression resulting in muscle differentiation (2), and that class II histone deacetylase (HDAC) 4 and 5 bind to MEF2 and inhibit MEF2/MyoD transactivation activity and muscle differentiation (27). Calcium/calmodulin-dependent protein kinase induces muscle differentiation by phosphorylation of HDACs 4 and 5 and shuttling of the phosphorylated HDACs from nuclear MEF2·HDAC complex to the cytoplasm (28). It has been also shown that class II HDACs-repressed muscle differentiation can be overcome by treating cells with IGF1, which induces HDACs export from the nucleus (29). However, the mechanism by which IGF1 regulates HDACs 4 and 5 has not been well characterized. In this report, we have shown that the protein level and kinase activity of Akt2 were elevated during muscle differentiation. The up-regulation of Akt2 was because of MyoD induction of Akt2 promoter activity, whereas activated Akt2 might result from autocrine production of IGFs by myoblasts under differentiation medium condition (4). Nevertheless, elevated Akt2 during muscle differentiation could mediate IGFs signals to regulate HDACs 4 and 5 functions, even though a previous study showed that Akt1 did not phosphorylate HDACs 4 and 5 (28). Additional studies are required to define the mechanism of Akt2 activation of MyoD-MEF2 transcriptional activity.

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